AD_____

GRANT NUMBER DAMD17-94-J-4410

TITLE: Int-3 Oncogene in Normal and Neoplastic Breast Development

PRINCIPAL INVESTIGATOR: Jan K. Kitajewski, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Int-3 Oncogene in Normal and Neoplastic Breast Development

5. FUNDING NUMBERS
DAMD17-94-J-4410

6. AUTHOR(S)
Jan K. Kitajewski, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Elongation and branching of epithelial ducts is required for mammary gland development. Branching morphogenesis of mammary TAC-2 cells was used to examine Wnts, HGF, TGF-β and Notch receptors in branching morphogenesis. Wnt-1 induced the elongation and branching of epithelial tubules, and strongly cooperated with either HGF or TGF-β2 in this activity. The Notch4(int-3) mammary oncoprotein, inhibited the branching morphogenesis induced by HGF and TGF-β2. The minimal domain of Notch4(int-3) required consists of the CBF-1 interaction domain and the cdc10 repeats. Co-expression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 overrides the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling play opposite roles in mammary gland development. We describe genetic evidence indicating that *sel-10* is a negative regulator of *lin-12/Notch* mediated signaling in *C. elegans*. Sequence analysis shows that SEL-10 is a member of the CDC4 family of proteins with a potential human ortholog. Co-immunoprecipitation data indicate that *C. elegans* SEL-10 complexes with LIN-12 and with murine Notch4. We propose that SEL-10 promotes the ubiquitin-mediated turnover of LIN-12/Notch proteins.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Breast Cancer	Notch, Mammary Oncogene, Transformation, Int-3	111
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[signature] 9/23/97

PI - Signature Date

Int-3 Oncogene in Normal and Neoplastic Breast Development

Annual Report 9/29/1997

Table of contents

Introduction

I. Nature of the problem

In the past several years, it has been shown that oncogenes contribute to the pathobiology of breast cancer. Mutational activation of the *Notch4* proto-oncogene has been shown to contribute to experimental mammary gland tumorigenesis in mouse. Several human Notch family genes have also been implicated in human cancers. There is strong evidence that the Notch4/int-3 proteins regulate the cell fate decisions required for the morphogenesis and functional differentiation of the mouse mammary gland. Despite this evidence, work on the role of the *Notch4/int-3* gene in breast cancer is still in its infancy. Extensive studies on other Notch family members (lin-12/Notch) in organisms more tractable to genetic analysis such as Drosophila and C. elegans, demonstrates the evolutionary conservation of these proteins and their fundamental importance in cell fate decisions.

The proposed research will investigate the role of Notch4/int-3 proteins in the normal development of mammary gland and other tissues and study the biochemical properties that are important for int-3 transforming activity. This information will broaden our understanding about the events which control normal mammary gland development and how alterations in those events can lead to neoplastic growth of the mammary gland.

II. Background

Mouse mammary tumor virus induces breast cancer in mouse by insertional mutagenesis. In tumors, viral integration can result in activation of the *Notch4* proto-oncogene by promoter insertion and results in expression of a truncated gene product (int-3) (1). The nucleotide sequence of the truncated gene product revealed homology with the Notch/lin-12 gene family (2). The full length cDNA of the *int-3* gene has been successfully cloned, and indeed it bears all the hallmarks of a Notch/lin-12 family gene. We proposed to name the full length *int-3* gene *Notch4*, and reserve the term *int-3* for the truncated oncogenice form which is induced by retroviral insertion. Several lines of evidence confirm a role for int-3 in mammary tumorigenesis. Transfection of a recombinant *int-3* genomic DNA fragment, encoding the truncated oncoprotein, into the HC11 mouse mammary epithelial cell line induces anchorage-independent growth in soft agar (2). Expression of this same genomic fragment *in vivo* as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland

development and impaired differentiation (3), intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors (adenocarcinomas) (4). It has also been reported that the normal *int-3* gene is endogenously expressed in the mouse mammary gland (5).

cDNA sequence of the 6.5kb full length *Notch4* gene indicates that it is a bona fide Notch family member. The Notch/lin-12 protein family currently consist of eleven members, Notch (Drosophila) (6), lin-12 and glp-1 (C. Elegans) (7, 8, 9), Xotch (Xenopus) (10), Notch 1, 2, 3 and 4 (Mouse) (2, 11-15), Notch 1 and 2 (Rat) (16,17), NOTCH 1 and 2 (Human) (18, 19). These genes encode for transmembrane receptor proteins. The extracellular domain of Notch/lin-12 family members contains variable numbers of EGF (epidermal growth factor) like repeats and other cysteine rich repeats named lin-12/Notch repeats(26). The intracellular domain of all Notch/lin-12 family members contains several copies of a repeat sequence, named cdc10 or ankyrin repeat. The cdc10 repeats have recently been implicated as a protein-protein interaction domain. The intracellular domain of this family of proteins also contains a PEST sequence, a nuclear localization signal, and an opa repeat. PEST sequences are found in proteins which are rapidly degraded or may also represent potential phosphorylation sites. An opa repeat is a protein domain that is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor binding proteins (27). The Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. The fundamental importance of these genes during development has been demonstrated by genetic analysis of lin-12, Notch and Xotch mutants (20-24). In the mouse, null mutants of Notch 1 and 2 are lethal during embryonic development, although the exact cause for this premature death is not known (25).

Genetic and molecular analysis have identified several proteins that participate in Notch signaling. Drosophila *Delta* (28) and *Serrate* (29) and C. elegans *Lag-2* (30)and *Apx-1* encode a family of structurally related ligands for the Drosophila Notch and C.elegans lin-12 and glp-1 receptors respectively. These ligands are transmembrane proteins, containing EGF-like domains and a cysteine rich DSL (Delta-Serrate-Lag-2) domain within the extracellular part of the protein. Recently, mouse homologues of these ligands have been cloned, *Jagged-1* (31) and *Dll-1* (32). These ligands have been demonstrated to regulate Notch receptor activity through cell-cell interactions. The products of three Drosophila genes, deltex, disheveled and suppressor of hairless (Su(H)) have been shown to interact with the intracellular domain of Notch and may thus participate in the intracellular signaling pathway of Notch (33,34) Furthermore, genetic analysis has revealed similar phenotypes in certain Deltex and Notch mutants.

Deletion of the extracellular part of Notch, Xotch and lin-12 proteins results in a dominant gain of function mutation (20-22). The truncated gene product encoding for the intracellular part of the receptor exhibits constitutively activated protein function. The phenotype observed in this class of mutants suggests that the truncated gene products delay cell determination and thereby increase the proportion of uncommitted stem cells, leading to a prolonged lifetime of the cell or to a greater number of descendants (20-22). By analogy to the function of other Notch/lin-12 family members in lower organisms, one can speculate that delay in differentiation and accumulation of pluripotent proliferative stem cells would result in a growth advantage, thereby increasing the probability for secondary oncogenic mutations. This model would propose that Notch proteins contribute to oncogenesis by stimulating stem cell growth and blocking differentiation.

Studies on the Notch protein in Drosophila demonstrated that the intracellular domain of the Notch protein is translocated to the nucleus when a truncated Notch protein (corresponding to the intracellular domain of the protein) is expressed as a transgene in Drosophila embryos (20). Based on the hypermorphic effect of the deletion mutants, and on the presence of a nuclear translocation signal in the intracellular domain of the protein, a hypothetical model would be that ligand binding to the receptor would result in cleavage of the intracellular domain of the receptor and subsequent translocation to the nucleus, where it could interact with its substrate.

Notch/lin-12 gene family members have been implicated in human tumorigenesis. Alteration of NOTCH-1 (also named TAN-1) has been associated with a T lymphoblastic neoplasm (18). The mutation of the NOTCH-1 gene in T lymphoblastic lymphomas is caused by a translocation that results in expression of a truncated gene product. TAN-1 mutations are analogous to the *int-3* activating mutations as a result of MMTV insertion, as well as to the dominant gain of function mutations of Notch, lin-12 and Xotch. Furthermore, human NOTCH-1 and NOTCH-2 (also named hN) were found to be overexpressed in human cervical carcinomas (19).

III. Purpose

The overall goal of the work proposed here is to understand in molecular details the function of the Notch4/int-3 proteins in mammary epithelial cells and during mouse mammary gland development, with the long term goal of understanding the role of the *int-3* gene in mammary tumorigenesis.

Body

I. Technical Objectives

The overall goal of the work proposed here is to understand in molecular details of the function of the Notch4/int-3 proteins in mammary epithelial cells and during mouse mammary gland development.

We proposed three specific aims to investigate the mechanisms of the functions of Notch4/int-3 proteins in a relatively simple and biological context:

1. Compare the normal int-3 protein to activated int-3 oncoproteins

We intend to first determine the primary sequence of the normal int-3 protein and evaluate whether the normal *int-3* gene encodes a Notch-like transmembrane receptor. We will then compare the biochemical and physiological properties of the normal int-3 protein with that of truncated int-3 oncoproteins. Our aim is to determine what structural alterations lead to oncogenic activation of int-3 and to evaluate if activated int-3 proteins act at the cell surface or the nucleus.

2. Identifying proteins that interact with the intracellular domain of int-3.

To elucidate the molecular mechanisms of the oncogenic effect of activated int-3, we will identify proteins or protein complexes that interact with int-3. Three approaches will be used: (1) affinity purification of associated proteins, (2) library screening with radiolabled int-3 proteins, and (3) a yeast two-hybrid genetic screen for int-3 associated proteins.

3. Analysis of int-3 expression pattern in the murine mammary gland.

The fact that overexpression of activated int-3 in mouse mammary gland has profound effects on the development of the gland suggests a role of nomrla *int-3* gene products in mammary gland development. We will determine whether *int-3* gene is normally expressed in the mamamry gland. Molecular and immunohistochemical techniques will be used to evaluate where and when in the normal mammary gland the *int-3* gene is expressed.

To fullfill these specific aims, we have proposed five technical objectives for a period of four years.

Task 1: Characterize the full length *int-3* cDNA. (Months 1-12)

Task 2: Study the production, processing and subcellular localization of normal int-3 proteins and int-3 oncoprotein in mammary epithelial cells. (Months 3-18)

Task 3: Evaluate the transforming potential of int-3 proteins. (Month 9-18)

Task 4: Identify proteins that interact with the intracellular domain of int-3. (Month 12-48)

Task 5: Analysis of the int-3 expression pattern in the murine mammary gland. (Months 12-48)

II. Experimental Results

This annual report describes the progress we have made during the first 36 months of this grant, with an emphasis on the last 12 months. The progress reported here will be related to the original tasks set out in Statement of Work.

1. Characterize the full length *int-3* cDNA. (Months 1-12)

This task has been completely fulfilled within the proposed period of time.

The *int-3* oncogene was classified in the lin-12/Notch protein family solely on the basis of its homology to the intracellular part of the lin-12/Notch family members. Previously, there was no direct evidence that demonstrates that the full length *int-3* encodes for a transmembrane protein. By cloning the full length *int-3* cDNA we have demonstrated that the *int-3* gene encodes a transmembrane protein, homologous to the lin-12/Notch family of transmembrane proteins. We have proposed to name the full length gene *Notch4*, and reserve the *int-3* nomenclature when referring to the truncated and oncogenic form of the gene (Appendix A).

A. Cloning truncated *int-3.* We have cloned the truncated *int-3* gene that encodes for the intracellular part of the protein and corresponds to the int-3 mammary oncogene (Appendix A)

B. Cloning of the full length int-3 We have used the PCR based method of RACE (Rapid Amplification of cDNA Ends) (35,36) to clone sequences that are located 5' from the truncated *int-3* (the truncated *int-3* transcript is localized at the 3' end of the gene). To clone the *int-3* full length cDNA, we have screened a mouse lung cDNA library. This choice of cDNA library was based on our findings of the mRNA expression analysis of *int-3*. Initially, the probes that were used in this screening analysis were derived from the cloned truncated *int-3*, as well as from the clones obtained by RACE. Positive clones were purified and sequenced and used as probes in successive rounds of screening in order to obtain the full length cDNA of the *int-3* gene. The length of the full length*int-3* mRNA is approximately 6.5kb based on our Northern blot analysis. The full length *int-3* sequence has been analyzed for its homology with other lin-12/Notch family members, and has been shown to have a high overall homology to the other known mouse *Notch* 1, 2 and 3 genes. The predicted amino acid sequence encodes for a transmembrane protein with a intracellular domain containing six ankyrin repeats, a transmembrane domain, and a extracellular domain containing three Notch/lin-12 repeats and twenty nine EGF-like repeats (Appendix A).

C. Sequencing *int-3* cDNAs We have sequenced the full length gene of int-3. According to its DNA and amino acid sequences, the full length gene of int-3 is a bona fide Notch/lin-12 family gene. It bears all the typical tructural motifs found in Notch/lin-12 family genes.

2. Study the production, processing and subcellular localization of normal int-3 proteins and int-3 oncoprotein in mammary epithelial cells. (Months 3-18)

The *int-3* gene was discovered in mouse mammary tumors, induced by MMTV infection. Insertional of *Notch4* gene by MMTV results in the expression of a truncated protein product (int-3) that is able to transform mammary epithelium cells both *in vivo* and *in vitro*.

A. Epitope-tagging of int-3 proteins Before antibodies against the Notch4/int-3 proteins (aim 3) were available, we added an epitope to the Notch4 protein and its deletion mutants including int-3 (37). The epitope we have chosen is derived from the influenza HemAgglutinin (HA) protein and is recognized by a monoclonal antibody 12CA5 (38). cDNAs were cloned into phagemid vectors that contain the sequence encoding the HA epitope situated downstream. Single strands were generated from the phagemids and used in a site-directed mutagenesis protocol with an oligonucleotide designed to loop-out the sequence between the last codon of our proteins and the first codon of the HA tag, to create an in-frame fusion. The HA epitope was fused to the carboxy termini of all the Notch4/int-3 proteins. This method allowed us to detect ectopically expressed Notch4/int-3 fusion proteins in transfection experiments, using anti HA antibodies in immunoblot analysis (Appendix B).

B. Generation of int-3 expressing cell lines *int-3* and*int-3/HA* fusion constructs were sub-cloned into eukaryotic vectors such as pLNCX vector (39) and pQNCX vector (our unpublished data). These vectors utilize the cytomegalovirus immediate early promoter/enhancer to drive expression of the *int-3* gene and the retroviral LTR to drive expression of the *neo* gene, which confers resistance to the drug G418. We have also generated several control retroviral vectors, such as vectors expressing the LacZ gene, vectors expressing an unrelated HA tagged protein, and vectors expressing int-3 without the HA tag. This last construct allowed us to determine that the HA tagging does not interfere with the transforming activity of int-3 (see below). In order to evaluate the transforming potential of int-3, a mammary epithelial cell line programmed to express int-3 was generated by infection with retroviral expression vectors. High-titer helper free retroviral stocks were generated using the BOSC23 ecotropic virus packaging cell line (40), media was collected and used to infect the mammary epithelial cell lines TAC-2 (43). The int-3 stably infected

TAC-2 mammary epithelial cell lines express copious amounts of recombinant int-3 proteins as was determined by immunoblot analysis (Appendix B). The expression level of the int-3 recombinant proteins in TAC-2 cells can be increased by treatment of the cells with sodium butyrate (Appendix B). In addition, four int-3 deletion mutants were generated. The deletion mutants lack either the amino terminal end (-NT), the cdc-10 repeats (-cdc), the carboxy terminal end (-CT), or both the amino and carboxy terminal ends (-NT-CT) of int-3. All four int-3 deletion mutants were epitope tagged at the carboxy terminus. TAC-2 cell lines were generated to express the int-3 deletion mutants as determined by immunoblotanalysis (Appendix B).

C. Biochemical analysis of int-3(Notch4) proteins produced in cell lines. The deduced amino acid sequence of the full length Notch4 protein predicts that Notch4 is a putative transmembrane protein. We will investigate this hypothesis by studying the intracellular localization of the Notch4 protein. These studies may confirm the predicted transmembrane nature of the Notch4 protein. The biochemical properties of Notch4 will be investigated once mammary epithelial cell lines have been generated that are programmed to express Notch4. A full length Notch4 transcript was assembled and the Notch4 protein was detected in protein lysates of transiently transfected 293T cells (data not shown). The molecular weight of epitope tagged Notch4 is approximately 215 KD. Immunofluorescence experiments on 293T cells transiently transfected with epitope tagged Notch4, cells demonstrated plasma membrane staining (data not shown).

D. Subcellular localization of int-3 proteins The intracellular localization of truncated int-3 was investigated in transiently transfected cells (293T and HeLa cells). We have investigated the intracellular localization by indirect immunofluorescence (45) using the anti-HA antibodies, and have found nuclear localization of truncated int-3 (as reported in annual report 9/95). Several deletion mutants have been generated in order to determine which part of the truncated int-3 protein is responsible for its nuclear translocation. These initial studies pointed out two domains that may contain a nuclear translocation signal, one N-termnal and one C-termnal of the ankyrin repeats. Interestingly, deletion of the ankyrin repeats alone did not affect the nuclear localization. Preliminary data suggest that a protein consisting solely of the int-3 ankyrin repeats resides in the cytoplasm (data not shown). These studies will also allow us to investigate whether transformation coalesces with cytoplasmic or nuclear localization of the int-3 protein.

3. Evaluate the transforming potential of int-3 proteins. (Month 9-18)

A. Generate cell lines expressing int-3 proteins We have generated TAC-2 cell lines that stably express int-3 proteins (Appedix B).

B. Evaluate the transformed properties of int-3 expressing cell lines We have proposed to study mammary transformation by int-3, we have, instead established a biological assay for int-3 based on its ability to block ductal morphogenesis of cultured mammary epithelial cells. The biological activity of int-3 was studied in the TAC-2 mouse mammary epithelial cell line. TAC-2 cells were isolated based on their ability to undergo branching morphogenesis when grown in a collagen gel matrix (46). This differentiation of the TAC-2 cells into tree-like structures can be specifically induced by treatment with hepatocyte growth factor (HGF) (46). TAC-2 cells that express int-3 protein fail to undergo branching morphogenesis, when grown in the presence of HGF (Appendix B). Control cell lines, TAC-2 cells expressing Lac-Z, and TAC-2 cells expressing the mammary oncogene Wnt-1, were still able to differentiate into tree-like structures when induced with HGF (data not shown and Appendix B), suggesting that the int-3 induced block of differentiation is specific. To investigate which domain of the int-3 protein is responsible for the block of branching morphogenesis of the TAC-2 cells, we expressed the int-3 deletion mutants described above and examined their effects on TAC-2 cells (Appendix B). We also investigated the growth characteristics of the different TAC-2 cell lines and found no differences in growth rate (day 2) or post-confluence growth rate (day 6) between int-3 expressing TAC-2 cells and control TAC-2 cells or TAC-2 cells expressing Wnt-1, in the presence or absence of HGF (Appendix B). The growth of TAC-2 cells was measured by a commercially available biochemical assay. These experiments suggest that the int-3 oncoprotein prevents differentiation of mammary epithelial cells without affecting the growth characteristics of these cells. The above described data are reminiscent of the mammary gland phenotype of the *int-3* transgenic mice. These animals that overexpress the *int-3* oncogene in the mammary gland fail to develop a normal mammary gland (no branching morphogenesis) and the mammary epithelial cells present display an undifferentiated phenotype. In addition, our experiments also demonstrated that the int-3 and Wnt-1 oncoproteins have opposing activities in the branching morphogenesis pathway in TAC-2 mammary epithelial cells, and that the Wnt-1 oncoprotein can act as a morphogen (as opposed to mitogen) in the TAC-2 assay (Appendix B).

We have attempted to study the biological effects of Notch4 protein on TAC-2 cells. However, due to technical difficulties (*Notch4* gene is too large for retrovirus mediated gene transfer) we are still in the process of making stable TAC-2 cell lines expressing Notch4 proetin.

Once these cell lines are obtained, we will evaluate the biological activity of Notch4 in TAC-2 cells.

Since Notch4 is normally expressed in endothelial cells (Appendix A), We have generated stable cell lines expressing either int-3, Notch4, or Jagged-1 (a putative Notch ligand) using a rat brain endothelial cell line. In our preliminary experiments, an int-3 specific biological phenotype was observed (data not shown). And interestingly Jagged-1 gives exactly the same phenotype in this cell line but Notch4 seems to have no effect. These results suggest that this endothelial cell line may express an endogenous Notch (possibly Notch4) that can be activated by Jagged-1 and that Notch4 overexpression does necessarily result in a gain-of-function phenotype. These preliminary experiments will be repeated and further studied.

4. Identify proteins that interact with the intracellular domain of int-3. (Month 12-48)

A. Identify candidate proteins that may interact with the intracellular domain of int-3 (corresponds to task 4a, 4b, 4c) A genetic screen for genes that are involved in Notch/lin-12 signaling has been done in C. elegans by Dr. Iva Greenward at Columbia University. In collaboration with Dr. Greenwald's lab, we select C. elegans genes identified in their genetic screen according to their genetic and predicted biochemical features, as candidate genes that may encode int-3 binding proteins. The protein products of these candidate genes will be further tested in two-hybrid system and by biochemical and biological approaches. One candidate gene, C. elegans *sel-10*, a CDC4 family member has been shown in our assays to form a complex with C. elegans LIN-12 and mouse int-3 proteins (Appendix C). A human homologue of *sel-10* has been identified through genebank search (Appendix C), and its role in the Notch/lin-12 pathway is being investigated.

B. Identify int-3 binding proteins using yeast two-hybrid genetic screen (corresponds to task 4d) To study the downstream signaling pathway of Notch4 receptor, we planned to carry out yeast two hybrid screen to search for proteins that interact with the intracellular domain of Notch4. We first generated two bait constructs for two hybrid screen. They contain either the entire intracellular domain or the ankyrin repeats region fused to LexA or GAL4 DNA binding domain. Unfortunately, when we tested the two constructs for their ability to activate LacZ reporter gene expression in yeast on their own, the ankyrin repeat construct gave very robust positive signal while the other construct produced a medium level positive signal. We tried to tackle this problem with bait constructs containing a series of deletion mutants of the intracellular domain of int-3, hoping to get rid of the transcriptional activity of the bait yet keep the intracellular domain as much as

possible so that we might still identify Notch4 binding proteins through two hybrid screen. However, all the deletion mutant constructs we have tested are more or less active in β-galactosidase assay (data not shown). We tried different approaches to solve this technical difficulty, including testing our bait contructs in different yeast strains, testing the bait constructs for HIS3 reporter gene activation and adding 3-AT in yeast culture medium to suppress the leakiness of the HIS3 gene. However, under all the conditions we have tested, our bait constructs always display intrinsic transcriptional activity. These constructs, if used for two-hybrid screen, will inevitably cause high background. Therefore, they seem to be inappropriate for two-hybrid screen. But we still co-expressed the bait construct containing the entire intracellular domain with several of our candidate genes in yeast. Although the bait itself gives background expression of LacZ reporter gene, when it is co-expressed with some of our candidate genes, the signal is much stronger than the background. The same results were observed constantly in several independent experiments, suggesting that some of the C. elegans condidate proteins interact with the intracellular domain of Notch4. One of these genes, C. elegans sel-10 is further tested by biochemical approaches.

C. Characterize int-3 binding proteins (corresponds to task 4d) The interactions between C. elegans SEL-10 and LIN-12 or int-3 is confirmed by co-immunoprecipitation assay (Appendix C). Epitope-tagged (HA or myc) SEL-10 was co-transfected into 293T cells with either LIN-12intraHA or int-3. We then use antibodies to immunoprecipitate LIN-12intraHA or int-3 and probe for SEL-10 proteins. Our results indicate that the intracellular domains of LIN-12 or Notch4 exist in the same protein complex as SEL-10. These results are confirmed by immuoprecipitating SEL-10 first and then probing for the existence of LIN-12intraHA or int-3.

5 Analysis of the int-3 expression pattern in the murine mammary gland. (Months 12-48)

We proposed to analyze the expression pattern of the Notch4 protein as well as the *Notch4* mRNA. Such analysis will give us further insights in the function of the Notch4 protein. We planed to study the expression pattern of *Notch4* in different mouse tissues to determine if the Notch4 protein is expressed in a tissue specific manner, and we will investigate whether *Notch4* is expressed in the mammary gland and whether this expression is developmentally regulated.

A. Generate int-3 specific antibodies To study the biological and biochemical behavior of the Notch4 and int-3 proteins, we have successfully generated rabbit antiserum against a GST fusion protein of Notch4. An intracellular fragment of Notch4 protein, which is C-terminal to the ankyrin repeats, was fused to GST protein. This fragment is the least homologous domain among

different murine Notch proteins and therefore, it was chosen as immunogen to avoid cross-reactivity with other proteins. The specificity of the antiserum as well as its ability to recognize Notch4/int-3 proteins in Western blot and immunoprecipitation assays has been determined (Appendix C).

B. Immunohistochemical analysis of int-3 proteins in mammary tissue We are in the process of using the polyclonal antibodies against Notch4 in immunohistochemistry experiments. Preliminary data have confirmed the endothelial cell specific expression of Notch4 in adult mouse kidney glomeruli (data not shown). These experiments will be repeated and optimized in order to perform a detailed analysis of Notch4 expression in adult mouse tissues such as the developing mammary gland.

C. Northern blot analysis of int-3 expressed in mammary tissue We have not analyzed mammary gland expression but have determined the expression pattern of Notch4/int-3 in adult and embryonic tissues. *Notch4* mRNA expression was studied by Northern blot analysis and in situ hybridization using probes derived from the 3' UTR (Appendix A). *Notch4* gene encodes for a 6.5 kb transcript that is highly expressed in lung, heart and kidney in adult tissues. Several shorter *int-3* transcripts were observed in adult testis, and were later shown to be the products of aberrant transcriptional events in post-meiotic spermatids (Appendix A). The *Notch4* transcript is expressed at all stages of mouse development (day 6.5 to 15.5) (data not shown).

D. In situ hybridization analysis of int-3 RNA in mammary tissue In situ hybridization (using the same probe as in the Northern blot analysis) was performed to determine the cellular origin of *Notch4* expression during mouse development, and revealed endothelial specific expression. In situ hybridization on adult lung tissue was performed and revealed endothelial cell specific expression of *Notch4*. (Appendix A).

Conclusions

Data presented in this annual report represent our progress in the experiments outlined in the specific aims of the research proposal. As outlined in the statement of work in the research proposal, we have largely completed the aims as scheduled for months 1-36.

The cloning of *int-3/Notch4* is completed. The deduced amino acid sequence of the Notch4 protein displays high homology with the *Notch/lin-12* gene family members, identifying this gene as the fourth murine *Notch* gene. We have proposed to rename this gene *Notch4*, reserving the *int-3* nomenclature for the activated and truncated form. The deduced amino acid sequence of Notch4 reveals 29 EGF-like repeats and 3 Notch/lin-12 repeats in the extracellular domain, a transmembrane domain, and an intracellular domain containing six ankyrin repeats. The above data confirms the initial hypothesis that *int-3* is a bona fide member of the *Notch/lin-12* gene family.

The truncated *int-3* gene and the full length *Notch4* gene has been assembled, epitope tagged, and cloned into an eukaryotic expression vector. Cell lines have been generated that are programmed to express Notch4/int-3 proteins. A biological assay in TAC-2 cells was was used to evaluate int-3 activity. In this assay, the int-3 oncoprotein can prevent growth factor mediated differentiation, in an analogous matter to the *int-3* transgenic mouse phenotype. Another biological assay in an endothelial cell line is being developed.

Sel-10, a negative regulator of the *Notch/lin-12* siganling pathway has been identified in C. elegans. We have shown SEL-10 proteins complexes with both LIN-12 and Notch4, indicating the regulatory function of *Sel-10* is conserved among different species. A human homologue of *Sel-10* has been identified and is under investigation.

The expression analysis of the *Notch4 mRNA* was analyzed in adult tissues as well as during mouse development. The *Notch4* transcript consists of a 6.5 kb mRNA species, that is specifically expressed in embryonic and adult endothelial cells. A rabbit polyclonal antibody was successfully raised against the intracellular domain of Notch4, and the specificity and immune titer was determined.

References

1. Gallahan, D. and Callahan, R. Mammary Tumorigenesis in Feral Mice: Identification of a New int Locus in Mouse Mammary Tumor Virus (Czech II)-induced Mammary Tumors. *J. Virol.* 61, 66-74 (1987)

2. Robbins, J., Blondel, B.J., Gallahan, D. and Callahan, R. Mouse Mammary Tumor Gene int-3: a Member of the Notch Gene Family Transforms Mammary Epithelial Cells. *J.Virol.* 66, 2594-2599 (1992)

3. Smith, G.H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G. and Gallahan, R. Constitutive expression of a truncated int-3 gene in mouse mammary epithelium impairs differentiation and functional development. *Cell Growth & Diff.* 6, 563-577 (1995)

4. Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G.H., Merlino, G. and Callahan, R. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Devel.* 6, 345-355 (1992)

5. Sarkar, N.H., Haga, S., Lehner, A.F., Zhao, W., Imai, S. and Moriwak, K. Insertionsal mutaion of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: Normal- and Tumor-tissue specific expression of *int-3* transcripts. *Virology* 203, 52-62 (1994)

6. Wharton, K.A., Johansen, K.M., Xu, T. and Artavanis-Tsakonis, S. Nucleotide Sequence from the Neurogenic Locus Notch Implies a Gene Product That Shares Homology with Proteins containing EGF-like repeats. *Cell* 43, 567-581 (1985)

7. Yochem, J., Weston, K. and Greenwald, I. The Caenorhabditis elegans lin-12 gene encodes a transmembrane protein with overall similarity to Drosophila Notch. *Nature* 335, 547-550 (1988)

8. Greenwald, I. lin-12, A Nematode Homeotic Gene, Is Homologues to a Set of Mammalian Proteins That Includes Epidermal Growth Factor. *Cell* 43, 583-590 (1985)

9. Yochem, J. and Greenwald, I. glp-1 and lin-12, Genes Implicated in Distinct Cell-Cell Interactions in C. elegans, Encode Similar Transmembrane Proteins. *Cell* 58, 553-563 (1989)

10. Coffman, C., Harris, W. and Kintner, C. Xotch, the Xenopus Homolog of Drosophila Notch. *Science* 249, 1438-1441 (1990)

11. Lardelli, M. and Lendahl, U. Motch A and Motch B-Two Mouse Notch Homologues Coexpressed in a Wide Variety of Tissues. *Exp.Cell Res.* 204, 364-372 (1993)

12. Del Amo, F., Smith, D.E., Swiatek, P.J., Gendron-Maguire, M., Greenspan, R.J., McMahon, A.P. and Gridley, T. Expression pattern of Motch, a mouse homolog of Drosophila Notch, suggests an important role in early postimplantation mouse development. *Development* 115, 737-744 (1992)

13. Del Amo, F., Gendron-Maguire, M., Swiatek, P.J., Jenkins, N.A., Copeland, N.G. and Gridley, T. Cloning, Analysis, and Chromosomal localization of Notch-1, a Mouse Homolog of Drosophila Notch. *Genomics* 15, 259-264 (1993)

14. Reaume, A.G., Conlon, R.A., Zirngibl, R., Yamaguchi, T.P. and Rossant J. Expression Analysis of a Notch Homologue in the Mouse Embryo. *J.Dev.Biol.*154, 377-387 (1992)

15. Lardelli, M., Dahlstrand, J. and Lendahl, U. The novel Notch homologue mouse *Notch* 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech Devel* 46, 123-136 (1994)

16. Weinmaster, G., Roberts, V.J. and Lemke, G. A homolog of Drosophila Notch expressed during mammalian development. *Development* 113, 199-205 (1991)

17. Weinmaster, G., Roberts, V.J. and Lemke, G. Notch 2: a second mammalian Notch gene. *Development* 116, 931-941 (1992)

18. Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D. and Sklar,J. TAN-1, the human homolog of the Drosophila Notch gene, is broken by Chromosomal Translocations in T Lymphoblastic Neoplasms. *Cell* 66, 649-661 (1991)

19. Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L. and Artavanis-Tsakonis, S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc.Natl.Acad.Sci.* USA 92, 6414-6418 (1995)

20. Struhl, G., Fitzgerald, K. and Greenwald, I. Intrinsic Activity of the Lin-12 and Notch Intracellular Domains In Vivo. *Cell* 74, 331-345 (1993)

21. Coffman, C.R., Skoglund, P., Harris, W.A. and Kintner, C.R. Expression of an Extracellular deletion of Xotch Diverts Cell Fate in Xenopus Embryos. *Cell* 73, 659-671 (1993)

22. Fortini, M.E., Rebay, I., Caron, L.A. and Artavanis-Tsakonas S. An activated Notch receptor blockscell-fate commitment in the developing Drosophila eye. *Nature* 365, 555-557 (1993)

23. de Celis, J.F., Mari-Befa, M. and Garcia-Bellido, A. Cell-autonomous role of Notch, an epidermal growth factor homologue, in sensory organ differentiation in Drosophila. *Proc.Natl.Acad.Sci.* USA 88, 632-636 (1991)

24. Lieber, T., Kidd, S., Alcamo, E., Corbin, V. and Young, M.W. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Devel* 7, 1949-1965 (1993)

25. Swiatek, P.J., Lindsell, C.E., Franco del Amo, F., Weinmaster, G. and Gridley, T. Notch 1 is essential for postimplantation development in mice. *Genes Devel.* 8, 707-719 (1994)

26. Stifani, S., Blaumueller, C.M., Redhead, N.J., Hill, R.E. and Artavanis-Tsakonas, S. Human homologs of a Drosophila Enhancer of Split gene product define a novel family of nuclear proteins. *Nature* Genetics 2, 119-127 (1992)

27. Wharton, K.A., Yedvobnick, B., Finnerty,V.G. and Artavanis-Tsakonas, S. Opa: a novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in Drosophilla melanogaster. *Cell* 40, 55-62 (1985)

28. Kooh, P.J., Fehon, R.G. and Muskavitch, A.T. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. *Development* 117, 493-507 (1993)

29. Fleming, R.J., Scottgale, T.N., Diederich, R.J. and Artavanis-Tsakonis, S. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster. Genes Devel* 4, 2188-2201 (1990)

30. Henderson, S.T., Gao, D., Lambie, E.J. and Kimble, J. *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans. Development* 120, 2913-2924 (1994)

31. Lindsell, C.E., Shawber, C.J., Boulter, J. and Weinmaster, G. Jagged: A mammalian ligand that activates Notch 1. *Cell* 80, 909-917 (1995)

32. Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J-L. and Gossler, A. Transient and restricted expression during mouse embryogenesis of *DLL 1,* a murine gene closely related to Drosophila Delta. *Development* 121, 2407-2418 (1995)

33. Diederich, R.J., Matsuno, K., Hing, H. and Artavanis-Tsakonis, S. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* 120, 473-481 (1994)

34. Matsuno, K., Diederich, R.J., Masahiro, J.G., Blaumueller, C.M. and Artavanis-Tsakonis, S. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* 121, 2633-2644 (1995)

35. Ohara, O., Dorit, R.L. and Gilbert, W. One-sided polymerase chain reaction: the amplification of cDNA. *Proc.Natl.Acad.Sci.USA* 86, 5673-5677 (1989)

36. Apte, A.N. and Siebert, P.D. Anchor-ligated cDNA libraries: a technique for generating a cDNA library for the immediate cloning of the 5' ends of mRNAs. *Bio. Techniques* 15, 890-893 (1993)

37. Kolodziej, P.A. and Young, R.A. Epitope tagging and protein surveillance. *Methods Enzymol.* 194, 508-519 (1991)

38. Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L. and Lerner, R.A. The structure of an antigenic determinant of a protein. *Cell* 37, 767-778 (1984)

39. Miller, A.D. and Rosman, G.J. Improved retroviral vectors for gene transfer and expression. *Bio. Techniques* 7, 980-990 (1989)

40. Pear, W.S., Nolan, G.P., Scott, M.L. and Baltimore, D. Production of high titer helper-free retrovirus by transient transfection. *Proc.Natl.Acad.Sci.* USA 90, 8392-8396 (1993)

41. Vaidya, A.B., Lasfargues, E.Y., Sheffield, J.B. and Coutinho, W.G. Murine mammary tumor virus (MMuTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology* 90, 12-22 (1978)

42. Danielson, K.G., Oborn, C.J., Durban, E.M. and Medina, D. Mouse mammary epithelial cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proc.Natl.Acad.Sci.* USA 81, 3756-3760 (1984)

43. Soriano, J.V., Pepper, M.S., Nakamuro, T., Orci, L. and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J Cell Sci.* 108, 413-430 (1995)

44. Soule, H.D., Vazquez, J., Long, A., Albert, S. and Brennan, M. A human breast cancer line from a pleural effusion derived from breast carcinoma. *J. Natl. cancer Inst.* 51, 1409-1413 (1973)

45. Osborn, M. and Weber, K. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: Tubulin containing structures. *Methods Cell Biol.* 24, 97-132 (1982)

46. Soriano, J.V., Pepper, M.S., Nakamura, T., Orci, L. and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary epithelial cells. *J Cell Science* 108, 413-430 (1995)

Appendices

Appendix A
pages 23-31

Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. 1996.*Notch4/int-3*, a mammary proto-oncogene, is an endothelial cell-specific *Notch* gene. *Development,* 122: 2251-2259.

Appendix B
pages 32-73

Uyttendaele, H., J. Soriano, R. Montesano, and J. Kitajewski. 1997. Notch and Wnt proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion. Submitted.

Appendix C
pages 74-111

Hubbard, E.J.A., G. Wu, J. Kitajewski and I. Greenwald. 1997. *sel-10*, a negative regulator of *lin-12* activity in *C. elegans*, encodes a member of the CDC4 family of proteins. *Genes Dev.*, in press.

Development 122, 2251-2259 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV4679

Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene

Hendrik Uyttendaele[1], Giovanna Marazzi[2], Guangyu Wu[1], Qingyou Yan[1], David Sassoon[2] and Jan Kitajewski[1,*]

[1]Department of Pathology in the Center of Reproductive Sciences, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
[2]Brookdale Center for Molecular Biology, Mount Sinai Medical Center, New York, NY, 10029, USA

*Author of correspondence (e-mail: jkk9@columbia.edu)

SUMMARY

The *int-3* oncogene was identified as a frequent target in Mouse Mammary Tumor Virus (MMTV)-induced mammary carcinomas and encodes the intracellular domain of a novel mouse *Notch* gene. To investigate the role of the *int-3* proto-oncogene in mouse development and carcinogenesis, we isolated cDNA clones corresponding to the entire coding potential of the *int-3* proto-oncogene. We propose to name this gene *Notch4* and reserve the *int-3* nomenclature for references to the oncogenic form. The deduced amino acid sequence of Notch4 contains conserved motifs found in Notch proteins; however Notch4 has fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than other mouse Notch homologues. Comparison of the coding potential of the *int-3* gene to that of *Notch4* suggests that loss of the extracellular domain of Notch4 leads to constitutive activation of this murine Notch protein. In situ hybridization revealed that *Notch4* transcripts are primarily restricted to endothelial cells in embryonic and adult life. Truncated *Notch4* transcripts were detected in post-meiotic male germ cells. The distinct Notch4 protein features and its restricted expression pattern suggests a specific role for *Notch4* during development of vertebrate endothelium.

Key words: Notch, int-3, endothelial cells, mammary oncogene

INTRODUCTION

The *int-3* gene was originally identified on the basis of its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor-specific transcripts derived from the *int-3* gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 oncoprotein in mouse mammary epithelial cells (HC11) promotes anchorage-independent growth (Robbins et al., 1992). Expression of *int-3* as an MMTV-LTR-driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the *Notch/lin-12* gene family were first identified in *Drosophila* and *Caenorhabditis elegans* through genetic analysis of mutations that alter cell fate decisions (for reviews see Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). *Drosophila Notch* regulates multiple cell fate decisions that involve cell-cell interactions during fly development, for instance, control of cell fate decisions involving neural/epidermal specification in proneural clusters (Artavanis-Tsakonas and Simpson, 1991). The *C. elegans* lin-12 and glp-1 proteins are structurally related to Notch and are also involved in cell fate specifications during development in the nematode (Greenwald, 1985; Yochem and Greenwald, 1989). Genetic analysis of *Notch/lin-12* genes suggests that this family of genes controls binary cell fate decisions and inductive signaling that depend on cell-cell interactions (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Greenwald and Rubin, 1992). Alternatively, *Notch/lin-12* genes have been proposed to block cell differentiation, thus maintaining the competence of cells for subsequent cell-fate determination (Coffman et al., 1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins characterized by highly repeated, conserved domains. The amino terminus of Notch proteins encodes the extracellular domain and contains as many as 36 repeats of an EGF-like motif involved in ligand binding (Rebay et al., 1993) and three tandem copies of a Notch/lin-12 sequence motif of unknown function. The intracellular portion of Notch proteins is characterized by six tandem copies of a cdc10/ankyrin motif, thought

to be a protein-protein interaction domain (Michaely and Bennett, 1992) and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia, and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to *Drosophila Notch*, additional *Notch*-related genes have been isolated from mammals, including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991) and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et al., 1994). To date, three *Notch* homologues, *Notch1*, *Notch2* and *Notch3*, have been identified in the mouse, and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites and nervous system (Williams et al., 1995). Abundant expression of *Notch1*, *Notch2* and *Notch3* is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the *Notch1* gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death, preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

The gene products of *Drosophila Delta* (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990) and *C. elegans Lag-2* (Henderson et al., 1994; Tax et al., 1994) and *Apx-1* (Mello et al., 1994) are thought to act as ligands for Notch proteins. In the mouse, the orthologue of *Delta*, referred to a *Dll1* (*Delta-like gene 1*), is expressed during embryonic development in the paraxial mesoderm and nervous system in a pattern similar to that of mouse *Notch1* (Bettenhausen et al., 1995). A murine *Serrate*-related gene named *Jagged* has been identified and is partially co-expressed with murine *Notch* genes in the developing spinal cord (Lindsell et al., 1995).

We report here the identification and expression analysis of a fourth murine *Notch* homologue, which we propose to name *Notch4*, reserving the *int-3* nomenclature for the truncated oncogene. Although the intracellular domain of the int-3 oncoprotein shares homology with the Notch/Lin-12 protein family, we now provide a comparison of the full-length Notch4 protein with that of the int-3 oncoprotein. The activated int-3 protein contains only the transmembrane and intracellular domain of the Notch4 protein. The predicted amino acid sequence of Notch4 includes the conserved features of all Notch proteins, but Notch4 has seven fewer EGF-like repeats compared to Notch1 and Notch2 and contains a significantly shorter intracellular domain. Notch4 is expressed primarily in embryonic endothelium and in adult endothelium and male germ cells.

MATERIALS AND METHODS

Isolation and sequencing of *Notch4* cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5′ primer: CGTCCTGCTGCGCTTCCTTGCA and 3′ primer: CCGGTGCCTAGTTCAGATTTCTTA) designed from the *int-3* cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned *int-3* oncogene. Two consecutive 5′ RACE reactions (5′-Amplifinder RACE kit, Clonetech) using testis and lung cDNA were done to obtain cDNA clones located 5′ of the *int-3* oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene) and the TA cloning vector (Invitrogen) and used to generate probes to screen a lung cDNA library (Clonetech). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% formamide, 3× SSC, 100 mM Tris-HCl (pH 7.4), 5× Denhardt's solution, 0.2% SDS and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1× SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5′ restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full-length *Notch4* cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems). Sequence data from both strands were obtained for the entire *Notch4* cDNA and were analyzed and assembled using computer software (MacVector, Assemblylign).

Northern blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and northern blot hybridization analysis was performed. 20 μg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes, Stratagene) by capillary blotting. ^{32}P-labeled riboprobes were transcribed (Maxiscript in vitro transcription kit, Ambion) from *Notch4* cDNA clones encoding the 5′ or 3′ UTR (untranslated region) or ORF (open reading frame). The 3′ UTR *Notch4* cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 20 mM NaH_2PO_4 (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 10 μg/ml poly(A) mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2× SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2× SSC and 1% SDS. Membranes were exposed to X-ray film (X-OMAT AR, Kodak). The integrity of the RNA, as well as comparable amounts of RNA, were tested by rehybridization with a GAPDH probe.

In situ hybridization

Staged embryos ranging from 9 days post-coitum (d.p.c.) to birth were obtained from timed breedings of CD-1 mice. The morning when the the vaginal plugs appeared was counted as 0.5 d.p.c. Lungs was obtained from adult CD-1 mice. Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak emulsion and exposed for 2 weeks at 4°C. Analyses were carried out using both light- and dark-field optics on a Leica DA microscope. To avoid potential cross-hybridization with homologous RNAs, we used an antisense ^{35}S-labeled RNA probe corresponding to the 3′ UTR of *Notch4*. Probes were used at a final concentration of 9×10^4 dpm/ml.

RESULTS

Isolation and analysis of *Notch4* cDNA clones

The *int-3* mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full-length *int-3* gene, which we will refer to as *Notch4*, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, we have cloned cDNAs containing the complete coding potential of the *Notch4* gene. Using primers derived from the published sequence of the *int-3* oncogene, RT-PCR was used to isolate a 2.4 kb *int-3* cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal *int-3* gene, cDNAs were isolated by 5′ RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of *Notch4* transcripts detected by northern blot analysis; thus, we believe the cloned cDNA represents the full-length *Notch4* gene.

Several differences (insertions, deletions and single nucleotide changes) were found between the nucleotide sequence of *Notch4* reported here and the previously published *int-3* nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or, possibly, of mutations found in the tumor-derived *int-3* transcript (Robbins et al., 1992) that are not found in the *Notch4* gene. The nucleotide sequence of mouse *Notch4* has been deposited with GenBank under the Accession number U43691.

Analysis of the deduced *Notch4* amino acid sequence

Analysis of the deduced amino acid sequence of *Notch4* reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins and 58% similar and 40% identical to *Drosophila* Notch. Lower homologies were found when compared with the *C. elegans* lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). The N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site

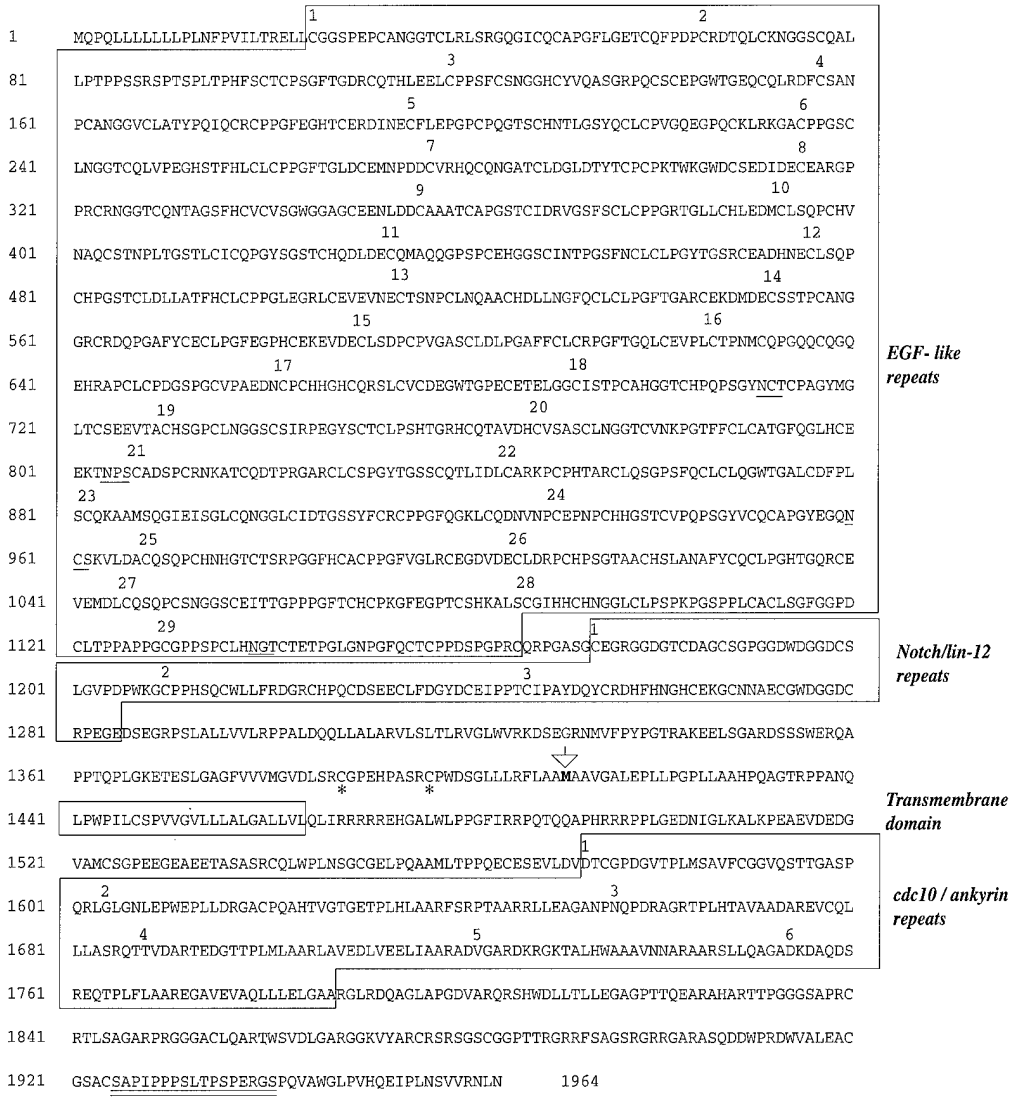


Fig. 1. Deduced amino acid sequence of Notch4 (GenBank accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, as follows: 29 epidermal growth factor(EGF)-like repeats; 3 Notch/lin12 repeats; a transmembrane domain; and 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.

was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane-spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992) and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in Notch proteins as equivalent EGF-like repeats. We analyzed the relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein. Fig. 2 schematizes the relationship of EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2, EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2 and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeat derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). For a discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2), see Lardelli et al. (1994).

EGF-like repeats 11 and 12 of *Drosophila* Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins in vitro (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig. 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteines of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins. In addition, EGF-like repeats 22-23 of Notch4 have been conserved among murine Notch proteins (EGF-like repeats 28 and 29 of Notch1) and equivalent EGF-like repeats in *Drosophila* Notch are implicated in the regulation of Notch protein function through genetic analysis of the *Abruptex* alleles of Notch (Kelley et al., 1987).

Notch4 also contains three Notch/lin-12 repeats, which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4 are two cysteines at positions 1388 and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

The intracellular domain of Notch4 contains the six ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52% and 55% identical to the ankyrin repeat domains of Notch1, Notch2 and Notch3, respectively. In all Notch proteins the number of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1), such as that found in *Drosophila* Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant homology to other known proteins. This C terminus is also much shorter in Notch4 (177 residues), than in other Notch proteins (457 residues in Notch1, 437 in Notch2 and 329 in Notch3).

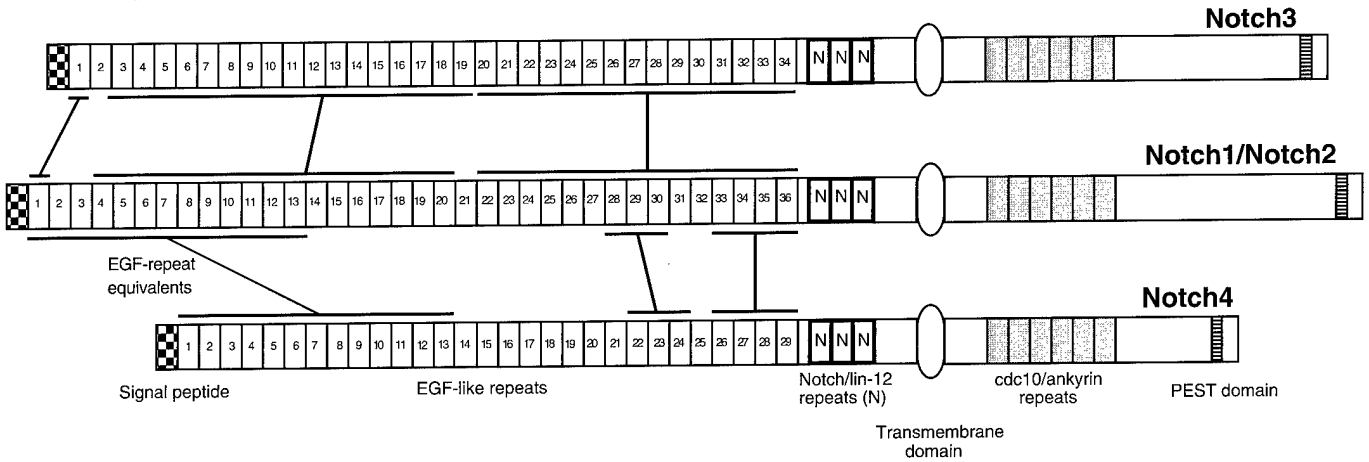


Fig. 2. Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified, connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains seven EGF-like repeats, fewer than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2, creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy terminus is shorter when compared to Notch1, 2 and 3.

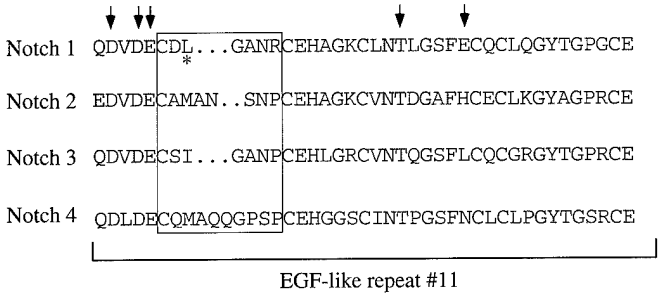


Fig. 3. Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium-binding sites are marked with arrows. A region within EGF-like repeat #11 of the Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in Xenopus Notch that obliterates binding to Delta is marked with an asterisk (*).

Analysis of *Notch4* transcripts in adult tissues

Several adult tissues were examined for the presence of *Notch4* transcripts by northern blot analysis. To minimize cross-hybridization with other mouse *Notch* transcripts, we used a riboprobe derived from the 3′ UTR of *Notch4*. In most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned *Notch4* cDNA. The 6.7 kb transcript is most highly expressed in lung, at lower levels in heart and kidney and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues, including brain, intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, *Notch4* expression varies widely in adult tissues. Other than in testis, we did not detect transcript size variation in different tissues.

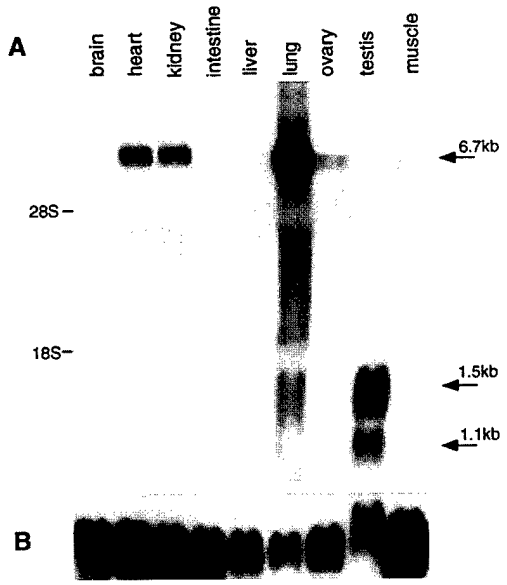


Fig. 4. Expression analysis of *Notch4* in adult mouse tissues. (A) Northern blot using a riboprobe transcribed from the 3′ UTR of *Notch4* (probe D in Fig. 5). (B) The same blot reprobed with a GAPDH probe. The transcript sizes of 6.7 kb, 1.5 kb and 1.1 kb are indicated and were estimated with reference to 28 S and 18 S rRNA migration.

Analysis of testis-specific truncated *Notch4* transcripts

To determine the cell lineage specificity of *Notch4* expression in the murine testis, RNA was analyzed in the germ cell-deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^v) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli and peritubular myoid cells (Mintz and Russell, 1957). Heterozygous litter mates (*W*/+) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^v) and testes with normal germ cells [*W*/+ and adult (+/+)] was done using a riboprobe derived from the 3′ UTR (probe D in Fig. 5C). Transcripts of 1.5 kb and 1.1 kb were detected in RNA from the testes of adult wild type and *W*/+ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

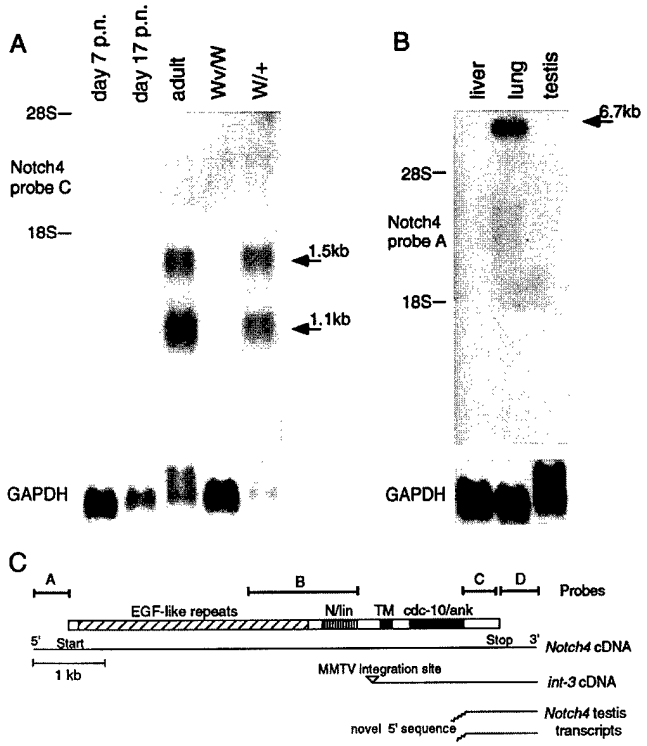


Fig. 5. Expression analysis of *Notch4* testis transcripts. (A) *Notch4* testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell-deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, W^v/W and *W*/+ mice, as indicated. (B) Northern blot analysis of several adult tissues with probe A, derived from the 5′ UTR of Notch4 and a GAPDH probe. (C) Schematic representation of truncated *Notch4* transcripts as compared to the full-length coding potential. Relative positions of probes used in the northern blot analysis are shown. Conserved elements of Notch family proteins are indicated. The MMTV integration site reported by Robbins et al. (1992) is indicated by an arrow. Novel 5′ sequences of testes cDNAs are indicated.

Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia, while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes (Nebel et al., 1961). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by northern blot hybridization to determine stage-specific expression of *Notch4* transcripts during male germ cell development. Both *Notch4* transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis (Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb *Notch4* transcripts is restricted to post-meiotic germ cells.

To determine the nature of the short *Notch4* transcripts in adult mouse testis, northern blot analysis was done using riboprobes derived from different regions of the *Notch4* coding sequence, as well as from 5′ and 3′ UTR (Fig. 5B). A riboprobe derived from the 5′ UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B), whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3′ UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Fig. 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3′ untranslated region of Notch4. Two independent clones of distinct size contained novel 5′ sequences unrelated to that found in the full-length *Notch4* cDNA (schematized in Fig. 5C, *Notch4* testis transcripts). Based upon the northern blot analysis described above and the sequence of the cloned testis cDNAs, we believe that *Notch4* transcripts are either derived from an alternate intronic promoter that is active in post-meiotic germ cells or that they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5′ untranslated region upstream of what we have currently identified. The predicted amino acid sequence of the testis *Notch4* transcripts with the novel 5′ sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode protein products. The testis transcripts may thus represent aberrant transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of *Notch4* during development and in adult lung

A 6.7 kb *Notch4* transcript was detected by northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and Sarkar et al., 1994) and adult lung (Fig. 4). To determine the spatial and temporal pattern of *Notch4* transcript accumulation during development, we examined mouse embryo tissue sections from 9.0 d.p.c. to birth using in situ hybridization. During embryonic development, as well as in postnatal tissues, *Notch4* is highly expressed in endothelial cells. Intense labeling for *Notch4* is observed in embryonic blood vessels at 9.0 d.p.c. (Fig. 6A,B). As shown in Fig. 6C,D, strong labeling is observed over the dorsal aorta, the aortic tract and the pulmonary artery in a 13.5 d.p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, we note that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6D,E) and no labeling is detected in the red blood cells

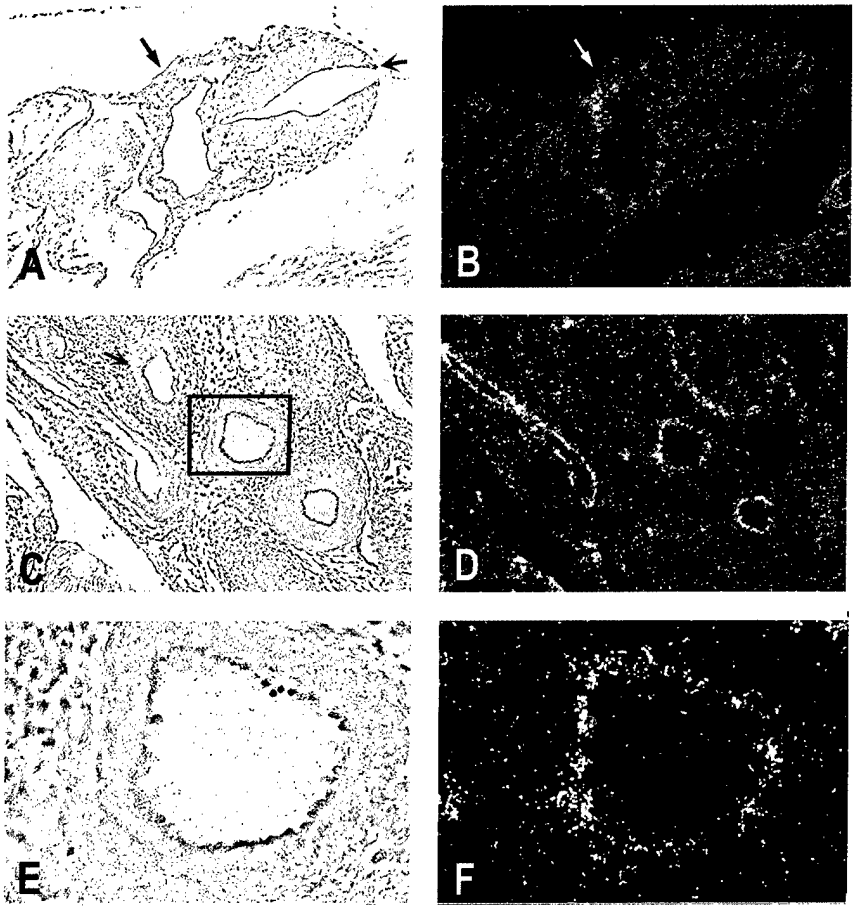


Fig. 6. *Notch4* is expressed in embryonic endothelial cells. (A,B) Phase contrast and dark-field photomicrograph of a horizontal section of a 9 d.p.c. embryo hybridized with a cRNA probe corresponding to *Notch4*. Strong labeling is detectable over the anterior cardinal vein (white/black arrows). Diffuse labeling is also present throughout the developing nervous system and at higher levels over the tip of the neural folds (red arrows). (C-F) Phase and darkfields images of a horizontal section of a 13.5 d.p.c. embryo hybridized for Notch4, showing the venous and arterial system anterior to the lung, including dorsal aorta arch, aortic and pulmonary tract. E and F are higher magnifications of the area framed in C. Embryonic vessels are labeled and, as shown in E and F, labeling is restricted to the endothelial cells lining the vessels. Arrows denote the gut, which does not have a detectable signal in the epithelium.

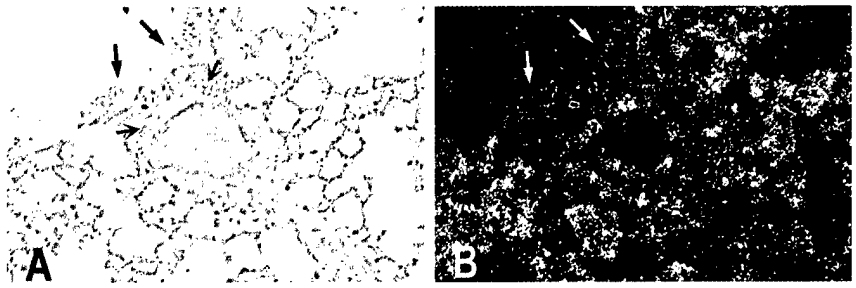


Fig. 7. *Notch4* is expressed in adult lung endothelial cells. (A,B) Phase contrast and dark-field photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to *Notch4*. Punctate staining is observed over the alveolar walls, which are predominantly composed of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).

in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 d.p.c. embryos. As shown in Fig. 6A,B, a light diffuse labeling is detected in the developing nervous system and a more distinct signal is observed at the tip of the neural folds. *Notch4* transcripts in the nervous system are still detectable at 11.5 d.p.c., but by 13.5 d.p.c. no labeling for *Notch4* is detectable in the nervous system (data not shown).

Since adult lung exhibited the highest levels of *Notch4* transcripts, in situ hybridization was performed on lung sections to determine whether *Notch4* expression remains endothelial cell-specific in adult life. Intense punctate staining was observed over the alveolar wall, indicative of capillary-specific expression (Fig. 7). The central component of the alveolar wall is the capillary flanked by pneumocyte type I epithelial cells, which line the alveolar lumen (Ross and Reith, 1985). Capillaries are highly localized in the alveolar wall and would give the punctate localized signal observed, as opposed to a more uniform pattern for epithelial cells lining the alveolar cavity. There is clearly no hybridization signal over other cellular components of the lung, that is, pseudostratified squamous epithelium, smooth muscle and connective tissue cells. The endothelial-specific expression probably underlies the abundance of *Notch4* transcripts found by northern blot analysis of highly vascularized adult tissues (lung, heart and kidney in Fig. 4).

DISCUSSION

We report here the identification of a novel mouse gene whose protein product exhibits structural homology with the vertebrate Notch protein family. We have named this gene *Notch4*, as it is the fourth murine *Notch* gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, but little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11, which has been proposed to be crucial for ligand binding. Structural variation in this repeat and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; *C. elegans* glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins, despite structural differences between them.

Notch4 is distinct from other Notch family proteins, based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of *Notch4*. This endothelial-specific expression of *Notch4* remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The *Notch4* expression pattern is in sharp contrast to the expression patterns of *Notch1*, *2* and *3*. These *Notch* genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). *Notch1* is the only other *Notch* gene reported to be expressed in endothelial cells (Reaume et al., 1992; Bettenhausen et al., 1995; Lindsell et al., 1995). Expression of *Notch1* and *4* in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development. Endothelial cell-specific expression has recently been reported for a putative Notch ligand, the chick *Serrate* homologue (Myat et al., 1996).

Since Notch proteins have been implicated in binary cell fate specification, regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebrafish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells, suggesting a common embryonic precursor which has been referred to as the 'hemangioblast'. The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. *Cloche*, *bloodless* and *spadetail* are mutants isolated in zebrafish that display phenotypes defective in either hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the *Flk-1* and the *Flt-1* genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995; Fong et al., 1995). Null mutants for the *Flk-1* gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the *Flt-1* gene display only hematopoietic cell development defects (Fong et al.,

1995). Mutational analysis of the *Notch4* gene in whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division, or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. In the normal mouse mammary gland, endogenous int-3 protein has been detected at low levels in mammary stroma and epithelium (Smith et al., 1995). Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in *int-3* transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins function are becoming understood through genetic studies in *Drosophila*. Deltex and Suppresser of Hairless [Su(H)] have been demonstrated to bind to the cdc10 repeats of the intracellular domain of *Drosophila* Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus. where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch proteins containing only the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993), suggesting a nuclear function for this domain. We have found that the int-3 oncoprotein, modified to encode a flu epitope-tag at the C terminus, is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). The activated int-3 protein lacks a signal peptide but contains a membrane-spanning domain and thus is not likely to enter the secretory pathway. This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

We show that the *int-3* gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described by Robbins et al. (1992), the oncogenic affects are probably the result of both overexpression or ectopic expression of *Notch4* mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural alterations reported to activate the effector function of *Drosophila* Notch and *C. elegans* lin-12 proteins (Greenwald, 1994) or oncogenic activation of TAN-1. Thus, loss of the extracellular domain is likely to lead to loss of the regulatory controls provided by the ligand-binding domain believed to reside in the EGF-like repeats of Notch4.

We would like to thank the DNA Core Facility of the Columbia University Cancer Center for help with sequencing; Kunsoo Rhee and Debra Wolgemuth for providing the mouse testis cDNA library; Zhili Zheng, Sara McGee and Marilyn Spiegel for technical assistance. We also thank Iva Greenwald, Debra Wolgemuth, Stephen Brown and Martin Julius for helpful discussions and comments. This work was supported by a grant to J. K. from the US Army Medical Research and Material Command (USAMRMC) under grant DAMD17-94-J-4069, by a pre-doctoral fellowship to H. U. from the USAMRMC under grant DAMD17-94-J-4153 and a Lucille Markey Fellowship and the Hirschl Foundation Award to D. S.

REFERENCES

Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the Notch locus. *Trends Genet.* **7**, 403-408.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J.-L. and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of *Dll*, a murine gene closely related to *Drosophila Delta*. *Development* **121**, 2407-2418.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of *Xotch* diverts cell fate in Xenopus embryos. *Cell* **73**, 659-671.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Davies, O. P. and Willison, K. R. (1993). Molecular mechanisms of differentiation in mammalian spermatogenesis. *Dev. Biol.* **3**, 179-188.

Diederich, R. J., Matsuno, K., Hing, H. and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120**, 473-481.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-661.

Fitzgerald, K., Wilkinson, H. A. and Greenwald, I. (1993). glp-1 can substitute for lin-12 in specifying cell fate decisions in *Caenorhabditis elegans*. *Development* **119**, 1019-1027.

Fleming, R. J., Scottgale, T. N., Diederich, R. J. and Artavanis-Tsakonas, S. (1990). The gene Serrate encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*. *Genes Dev.* **4**, 2188-2201.

Fong, G., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.

Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. *Nature* **365**, 555-557.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The suppresser of hairless protein participates in notch receptor signaling. *Cell* **79**, 273-282.

Franco Del Amo, F., Gendron-Maguire, M., Swiatek, P. J., Jenkins, N. A., Copeland, N. G. and Gridley, T. (1993). Cloning, analysis and chromosomal localization of *Notch-1*, a mouse homolog of *Drosophila Notch*. *Genomics* **15**, 259-264.

Gallahan, D. and Callahan, R. (1987). Mammary tumorigenesis in feral mice: identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J. Virol.* **61**, 66-74.

Goodbourn, S. (1995). Notch takes a short cut. *Nature* **377**, 288-289.

Greenwald, I. (1985). *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. *Cell* **43**, 583-590.

Greenwald, I. and Seydoux, G. (1990). Analysis of gain-of-function mutations of the lin-12 gene of *Caenorhabditis elegans*. *Nature* **346**, 197-199.

Greenwald, I. and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68**, 271-281.

Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. *Curr. Opin. Genet. Dev.* **4**, 556-562.

Handford, P. A., Mayhew, M., Baron, M., Winship, P. R., Campbell, I. D. and Brownlee, G. G. (1991). Key residues involved in calcium-binding motifs in EGF-like repeats. *Nature* **351**, 164-167.

Henderson, S. T., Gao, D., Lambie, E. J. and Kimble, J. (1994). *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development* **120**, 2913-2924.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signaling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merline, G. and Callahan, R. (1992). Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev.* **6**, 345-355.

Kelley, M.R., Kidd, S., Deutsch, W.A. and Young, M.W. (1987). Mutations altering the structure of epidermal growth factor-like coding sequences at the Drosophila *Notch* locus. *Cell* **51**, 539-548

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**, 2385-2396.

Lardelli, M. and Lendahl, U. (1993). *Motch A* and *Motch B*-two mouse *Notch* homologues coexpressed in a wide variety of tissues. *Exp. Cell Res.* **204**, 364-372.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel Notch homologue mouse Notch3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* **46**, 123-136.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

Maine, E. M., Lissemore, J. L. and Starmer, W. T. (1995). A phylogenetic analysis of vertebrate and invertebrate Notch-related genes. *Mol. Phylogen. Evol.* **4**, 139-149.

Marazzi, G. and Buckley, K. M. (1993). Accumulation of mRNAs encoding synaptic vesicle-specific proteins precedes neurite extension during early neuronal development. *Dev. Dynam.* **197**, 115-124.

Matsuno, K., Diederich, R. J., Go, M. J., Blaumeueller, C. M. and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121**, 2633-2644.

Mello, C. C., Draper, B. W. and Priess, J. R. (1994). The maternal genes apx-1 and glp-1 and establishment of dorsal-ventral polarity in the early C. elegans embryo. *Cell* **77**, 95-106.

Michaely, P. and Bennett, V. (1992). The ANK repeat: a ubiquitous motif involved in macromolecular recognition. *Trends Cell Biol.* **2**, 127-129.

Mintz, B. and Russell, E. S. (1957). Gene-induced embryological modifications of primordial germ cell in the mouse. *J. Exp. Zool.* **134**, 207-230.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.

Nebel, B. R., Amarose, A. P. and Hackett, E. M. (1961). Calendar of gametogenic development in the prepuberal male mouse. *Science* **134**, 832-833.

Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P. and Rossant, J. (1992). Expression analysis of a *Notch* homologue in the mouse embryo. *Dev. Biol.* **154**, 377-387.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multi-functional receptor. *Cell* **67**, 687-699.

Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74**, 319-329.

Robbins, J., Blondel, B. J., Gallahan, D. and Callahan, R. (1992). Mouse mammary tumor gene *int-3*, a member of the *notch* gene family transforms mammary epithelial cells. *J. Virol.* **66**, 2594-2599.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degrade proteins: The PEST hypothesis. *Science* **234**, 364-368.

Ross, M. H. and Reith, E. J. (1985). *Histology: A Text and Atlas,* pp. 526-527. Philadelphia: J. B. Lippincott Co.

Sarkar, N. H., Haga, S., Lehner, A. F., Zhao, W., Imai, S. and Moriwaki, K. (1994). Insertional mutation of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: normal- and tumor-tissue-specific expression of int-3 transcripts. *Virology* **203**, 52-62.

Sassoon, D. and Rosenthal, N. (1993). Detection of messenger RNA by in situ hybridization. *Methods Enzymol.* **225** (ed. P. M. Wassereman. and M. L. DePamphilis), pp. 384-404. San Diego: Academic Press, Inc.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X., Breitman, M. L. and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in FLK-1-deficient mice. *Nature* **376**, 62-66.

Smith, G. H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G. and Callahan, R. (1995). Constitutive expression of a truncated INT-3 gene in mouse mammary epithelium impairs differentiation and functional development. *Cell Growth Differ.* **6**, 563-577.

Stainier, D. Y. R., Weinstein, B. W., Detrich, H. W., Zon, L. I. and Fishman, M. C. (1995). cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**, 3141-3150.

Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S. (1992). Human homologs of a *Drosophila Enhancer of split* gene product define a novel family of nuclear proteins [published erratum appears in *Nature Genet.* (1992) Dec **2**(4), 343]. *Nature Genet.* **2**, 119-127.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

Sugaya, K., Fukagawa, T., Matsumoto, K., Mita, K., Takahashi, E., Ando, A., Inoko, H. and Ikemura, T. (1994). Three genes in the human MHC class III region near the junction with the class II: gene for receptor of advanced glycosylation end products, PBX2 homeobox gene and a notch homolog, human counterpart of mouse mammary tumor gene int-3. *Genomics* **23**, 408-419.

Swiatek, P. J., Lindsell, C. E., Franco del Amo, F., Weinmaster, G. and Gridley, T. (1994). *Notch 1* is essential for postimplantation development in mice. *Genes Dev.* **8**, 707-719.

Tax, F. E., Yeargers, J. J. and Thomas, J. H. (1994). Sequence of *C. elegans* lag-2 reveals a cell-signaling domain shared with Delta and Serrate of *Drosophila*. *Nature* **368**, 150-154.

Vassin, H., Bremer, K. A., Knust, E. and Campos-Ortega, J. A. (1987). The neurogenic gene *Delta* of *Drosophila melanogaster* is expressed in neurogenic territories and encodes for a putative transmembrane protein with EGF-like repeats. *EMBO J.* **6**, 3431-3440.

Weinmaster, G., Roberts, V. J. and Lemke, G. A. (1991). A homolog of *Drosophila Notch* expressed during mammalian development. *Development* **113**, 199-205.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1992). *Notch* 2: a second mammalian *Notch* gene. *Development* **116**, 931-941.

Williams, R., Lendahl, U. and Lardelli, M. (1995). Complementary and combinatorial patterns of *Notch* gene family expression during early mouse development. *Mech. Dev.* **53**, 357-368.

Yochem, J. and Greenwald, I. (1989). *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. *Cell* **58**, 553-563.

(Accepted 15 April 1996)

Notch and Wnt proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion.

Hendrik Uyttendaele[1], Jesus V. Soriano[2], Roberto Montesano[2] and Jan Kitajewski[1*]

[1]Department of Pathology in the Center for Reproductive Sciences, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
[2]Institute of Histology and Embryology, Department of Morphology, University Medical Center, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland

*Author of correspondence- Phone/fax (212) 305-3624

Running title: Notch and Wnt regulate branching morphogenesis

Key words: Notch4, int-3, Wnt-1, mammary oncogene, branching morphogenesis

Correspondent Footnote
Mailing address: Department of Pathology
Columbia University
630 West 168th Street
New York, NY 10032
Phone/Fax: (212) 305-3624
e-mail address: jkk9@columbia.edu

ABSTRACT

Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnts, HGF, TGF-β and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF-β2, and to strongly cooperate with either HGF or TGF-β2 in this activity. The Notch4(int-3) mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF-β2. The minimal domain within the Notch4(int-3) protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling may play opposite roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in Drosophila.

INTRODUCTION

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenic and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. Postnatal development of the mammary gland is influenced by gonadal hormones, with distinct developmental stages occurring during puberty, estrous, pregnancy and lactation. At birth, the mammary epithelial ducts have few side-branches. During puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, whereas the lateral buds differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which will produce milk at lactation. The mammary gland remodels after lactation ceases, and this process is characterized by the involution of the secretory lobules and regression to the ductal tree observed at puberty (review in (9, 38, 41)).

Mesenchymal-epithelial and epithelial-epithelial interactions are essential in the regulation of growth and development of the murine mammary gland. Peptide growth factors, such as Epidermal growth factor (EGF) (21), Fibroblast growth factors (FGF) (7), Hepatocyte growth factor (HGF), Insulin-like growth factor II (IGF-II) (3), Neuregulin (NRG) (51), and Transforming growth factor-β (TGF-β) (8, 37), have been implicated as regulators of mammary gland development based on their expression patterns and, in some cases, on their abilities to affect the development of the mammary gland. HGF (or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the mammary epithelial ducts at all stages (32). HGF can promote branching morphogenesis of the mammary ductal tree (32, 36, 47, 51) in several experimental settings. TGF-β1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation (8,

44). In vivo, TGF-β1 has been shown to inhibit ductal out-growth from the mammary end buds (27, 37). In vitro however, TGF-β1 has been shown to induce opposite effects depending on its concentration. TGF-β1 at high concentrations (0.5-5 ng/ml) inhibit ductal elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5-100 pg/ml) it is able to stimulate these biological processes (46).

The Wnt family of secreted growth factors are also implicated as regulators of the developing mouse mammary gland (34). *Wnt* genes are expressed during ductal development of the gland (*Wnt*-2, *Wnt*-5a, *Wnt*-7 and *Wnt*-10b) and during lobular development at pregnancy (*Wnt*-4, *Wnt*-5b and *Wnt*-6), and the expression of most *Wnt* transcripts is down regulated during lactation (16, 50). This pattern of expression during periods of morphogenesis has led to a proposed role for *Wnt* genes in morphogenic events during mammary gland development. *Wnt* gene expression has been documented in both the stromal and epithelial compartments of the mammary gland, raising the possibility of involvement in both stromal-epithelial and epithelial-epithelial interactions (5, 50). The *Wnt*-1 gene is not normally expressed within the mouse mammary gland, however its expression can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors (33). Mammary gland tumors develop in transgenic mice where ectopic *Wnt*-1 gene expression is controlled by the MMTV promoter; these mice display hyperplasia of the mammary epithelium and an increased incidence of tumors (48).

The *Notch4* gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (40, 42). The *Notch4* gene encodes for a large transmembrane receptor protein (14, 49). The int-3 oncoprotein is activated by MMTV insertion and corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted (49); this mutated version of Notch4 will be referred to as Notch4(int-3). In contrast to *Wnt*-1, expression of the

Notch4(int-3) oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop (25).

The aim of this study was to define the roles of both Wnt and Notch signaling in mammary gland ductal morphogenesis. Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF-β1 (46, 47), we demonstrate that activation of the Wnt and Notch signaling pathways has opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis whereas Notch4(int-3) inhibits branching morphogenesis by either HGF or TGF-β. Wnt-1 has the capacity to overcome the Notch4(int-3) mediated inhibition of branching morphogenesis.

MATERIALS AND METHODS

Reagents. Recombinant human HGF (rhHGF) was provided by Genentech, Inc. (San Francisco, CA). Recombinant TGF-β2 was provided by Dr. G. Gunderson (Columbia University, New York, NY). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal antibody (12CA5) was from Berkeley Antibody Co. (Richmond, CA) and HRP-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL).

cDNA clones. The murine *Notch4(int-3)* cDNA corresponds to a truncated *Notch4* cDNA; residues 4551 to 6244 of *Notch4* (Uyttendaele et al. 1996). An oligonucleotide encoding the haemagglutinin (HA) antigenic determinant was appended to the 3′ end of the *Notch4(int-3)* and *Wnt*1 cDNA's. Eighteen codons were added that specify the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine residue HA epitope (underlined). HA-tagged *Notch4(int-3)* and *Wnt*1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) with the coding region of the HA epitope situated downstream of the newly inserted cDNA. These two sequences were made co-linear by "loop-out" mutagenesis using oligonucleotides designed to eliminate the stop codon and non-coding 3′ sequence of the *Notch4(int-3)* and *Wnt*1 cDNAs. Oligonucleotides used in this procedure are as follows;

Notch4(int-3): CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG

Wnt-1: CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C .

The 5′ end of each oligo is complementary to the C-terminus of *Notch4(int-3)* or *Wnt*1 cDNA and their 3′ ends anneal to HA epitope-encoding sequence (underlined). Mutagenesis was done with the Muta-Gene*in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. *Notch4(int-3)* cDNA deletion mutants were generated from the epitope-tagged

Notch4(int-3) construct by restriction enzyme cloning, and were named ΔNT, ΔCDC, ΔCT and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the *Notch4* sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the *Notch4* sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the *Notch4* sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the *Notch4* sequence.

Cell culture. The TAC-2 cell line was derived from NMuMG cells as described previously (47). TAC-2 cell were grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The Bosc 23 retrovirus packaging cell line (Pear et al. 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 i.u./ml) and streptomycin (100ug/ml). Both cell lines were grown at 37°C in 8% CO_2.

Cell line generation. HA-tagged cDNAs were inserted into the retroviral vector pLNCX (28) wherein neomycin phosphotransferase (neo) expression is controlled by the murine leukemia virus LTR, and cDNA transcription is controlled by the cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX, however the neo gene is replaced by a hygromycin-resistence gene. Populations of TAC-2 cells, expressing either HA-tagged *Notch4(int-3)* or *Wnt1* cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting constructs into the BOSC 23 cell line by calcium phosphate co-precipitation, as previously described (35). Retroviral infection of TAC-2 cells was done by culturing cells with viral supernatants collected from transfected BOSC 23 cells two day post-transfection. Infections were done in the presence of 4 μg/ml polybrene for 12 hours after which medium was replaced to DMEM + 10% FCS. One day post-

infection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml Geneticin or 200 μg/ml hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in assays described below.

Collagen cell culture assays. TAC-2 cell lines were harvested using trypsin-EDTA, centrifuged, and embedded in three-dimensional collagen gels as previously described (47). Briefly, 8 volumes of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10x minimal essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4 x 10^4 cells/ml and 0.5 ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1ml of complete medium (DMEM + 10% FCS) with or without HGF or TGF-β2 was added to each well. TAC-2 collagen gel cultures were initially done in the presence and absence of 2mM sodium butyrate, but since no difference in phenotypes was observed, the sodium butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

Quantification of cord lenth and branching. TAC-2 cells were suspended at 5x10^3 or 1x10^4 cells/ml incollagen gels (500 μl) cast into 16-mm wells of 4-well plates (Nunc, Kampstrup, Rosklide, Denmark) and incubated in 500 μl complete medium in the presence or the absence of 10ng/ml HGF. After 7 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, and at least 3 randomly selected fields (measuring 2.2 mm x 3.4 mm) per experimental condition in each of 3 separate

experiments were photographed with a Nikon Diaphot TMD inverted photomicroscope. The total length of the cords present in each individual colony was measured with a Qmet 500 image analyzer (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as "0" in: a) colonies with a spheroidal shape, and b) slightly elongated structures in which the length to diameter ratio was less than 2. Quantification of branching was performed by counting all branch points in each colony. Values of cord length and branching obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of cords were out of focus and therefore could not be measured. Values were expressed either as mean cord length and number of branch points per photographic field (46) or as mean cord length and number of branch points per individual colony (47). The mean values for each experimental condition were compared to controls using the Student's unpaired T-test.

Immunoblot analysis. HA-tagged Notch4(int-3), Notch4(int-3) deletion mutants and Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and then removed from dishes in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose by electroblotting, and then blocked overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots

were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature. After four hours, the blot was washed three times for 5 min. each in TBST. Blots were exposed to a 1:16,000 dilution of HRP-conjugated sheep anti-mouse IgG. Blots were washed as above and then incubated 1-2 min. in enhanced chemiluminescence reagents (Amersham Inc, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

RESULTS

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular shaped cell aggregates to poorly branched structures. Under these same conditions, TAC-2 cells grown in the presence of either HGF or TGF-β1 develop an extensive network of branching cords that consist of elongated epithelial cords or tubules with multiple branch points (46, 47). This TAC-2 cell phenotype is thus reminiscent of the branching morphogenesis of epithelial ducts in the mammary gland and provides an experimental model to study the roles of growth factors in the development of the mammary gland. To investigate the role of Wnt and Notch signaling in mammary epithelial cell growth and morphogenesis we ectopically expressed either the Wnt-1 or activated Notch4(int-3) oncoproteins and analyzed their effects on branching morphogenesis of TAC-2 cells.

Wnt-1 stimulates TAC-2 cell branching morphogenesis. The biological activity of Wnt proteins was evaluated by generating TAC-2 cells ectopically expressing a *Wnt*-1 cDNA. TAC-2 cell lines programmed to express *Wnt*-1 (TAC-2 Wnt-1) were generated using the retroviral vector pLNCX to drive *Wnt*-1 expression from the CMV promoter. As a control, TAC-2 cells were generated that were programmed to express LacZ (TAC-2 LacZ). To evaluate the expression levels of Wnt-1 proteins in the cell lines generated, the *Wnt*-1 cDNA was fused at the carboxy terminus to the haemagglutinin-epitope (HA) tag, allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). Cell extracts from TAC-2 cell lines contained Wnt-1 proteins (Fig. 1) that migrated as a series of proteins with molecular weights between 41 and 45 kD, due to differential glycosylation. The ectopic expression of Wnt-1 proteins in TAC-2 Wnt-1 cells can be significantly increased by treating cells with sodium butyrate (2mM), which enhances transcription of the CMV promoter (Fig. 1). We found that addition of sodium butyrate to the TAC-2 branching morphogenesis assay did not

alter or enhance the TAC-2 cell phenotypes described below. The ensuing analysis of branching morphogenesis was conducted without sodium butyrate treatment.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for four days (Fig. 2A). Addition of either HGF (20 ng/ml) or TGF-β2 (50 pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Fig. 2B and 2C). We utilized TGF-β2 in our assays, which we found has an identical activity as TGF-β1 in the induction of branching morphogenesis of TAC-2 cells (46). When TAC-2 cells are programmed to express Wnt-1 proteins, cell colonies form cords with moderate branching even in the absence of exogenous growth factors (compare Fig. 2A and 2D). When TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (Fig. 2E and 2F), a highly extensive branching network is observed. This network of epithelial tubules is significantly more extensive when compared to control TAC-2 LacZ cells grown under identical conditions. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope tagged *Wnt*-1 cDNA or a HA epitope tagged *Wnt*-1 cDNA transcribed from a SV40 based retroviral vector (data not shown). Thus, Wnt-1 activity was confirmed in at least three independently produced TAC-2 cell lines.

The morphological analysis of TAC-2 cell cultures suggested that Wnt-1 cooperates with either HGF or TGF-β2 in the induction of branching morphogenesis. To characterize combined effects of Wnt-1 and HGF, quantitative evaluation of cord length and the number of branch points was conducted (Fig. 3A and 3B). Both analyses showed that the branched network formed by TAC-2 Wnt-1 cells grown under control conditions was comparable to that found for HGF-treated TAC-2 LacZ cultures. When TAC-2 Wnt-1 cells are grown in the presence of HGF, both cord length and number of branch points is significantly greater than the combined values for TAC-2 Wnt-1 cells without HGF and TAC-2 LacZ cells grown with HGF (Fig. 3A and 3B). Thus, Wnt-1

and HGF act in a cooperative fashion to induce branching morphogenesis of TAC-2 cells.

To determine whether the effects of Wnt-1 on TAC-2 cell branching morphogenesis are due to effects on the growth characteristics of TAC-2 cells we compared the growth of the TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen coated dishes, either in the presence or absence of HGF, and viable cell numbers were determined either two or six days after plating. No significant differences in cell number were found between control TAC-2, TAC-2 LacZ, or TAC-2 Wnt-1 cell lines grown in either the presence or absence of HGF (data not shown). When grown under these conditions, TAC-2 Wnt-1 cells and control TAC-2 cells both displayed contact inhibition at confluence and had similar morphological characteristics. Hence, the effects of Wnt-1 on TAC-2 branching morphogenesis are not correlated with mitogenic activity of Wnt-1 and are dependent on growth in three dimensional collagen gels. Identical results were obtained with TAC-2 cell lines that were programmed to express Wnt-1 using a SV40 based retroviral vector.

To further characterize morphogenic activities of Wnt-1 proteins, we analyzed TAC-2 cells induced to form cyst structures in collagen gel cultures. When TAC-2 LacZ cells are grown in collagen gels in the presence of hydrocortisone and cholera toxin, they form spheroidal cysts enclosing a widely patent lumen, as previously observed with non-transfected TAC-2 cells (47)(Fig. 4A). In contrast, under the same experimental conditions, TAC-2 Wnt-1 cells form branching structures (Fig. 4B). This clearly indicates that Wnt-1 expression modifies the spatial arrangement of TAC-2 cells and therefore has a morphogenic effect.

Notch4(int-3) inhibits TAC-2 cell branching morphogenesis. Notch activity in branching morphogenesis was evaluated by expressing an activated form of the Notch4 receptor, Notch4(int-3), in TAC-2 cells. TAC-2 cell lines programmed to express the Notch4(int-3) proteins were generated using the retroviral vector pLNCX and will be referred to as TAC-2 int-3. Notch4(int-3) was HA-epitope tagged at the carboxy terminus to allow detection of ectopically expressed proteins. Immunoblot analysis using anti-HA antibodies (Fig. 1) demonstrates that Notch4(int-3) proteins migrate with an approximately molecular weight of 60 kD, corresponding well to its predicted molecular weight. Notch4(int-3) expression is detected in extracts from TAC-2 int-3 cells and protein levels can be significantly increased by treatment with sodium butyrate (Fig. 1).

When TAC-2 int-3 cells are grown in collagen gels and incubated in the presence of either HGF or TGF-β2 (Fig 2H and 2I), cell colonies no longer form elongated cords like control cultures (Fig 2B and 2C). Instead, HGF- or TGF-β2-treated TAC-2 int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2 LacZ or TAC-2 int-3 colonies grown in the absence of HGF or TGF-β2 (Fig 2A and 2G). An identical phenotype was observed in at least three independently produced cell lines, including TAC-2 cells programmed to express a non-epitope tagged *Notch4(int-3)* cDNA or a HA epitope tagged *Notch4(int-3)* cDNA transcribed from a SV40 based retroviral vector (data not shown). Interestingly, we found that a smaller percentage of TAC-2 int-3 cells give rise to colonies in collagen gels with respect to TAC-2 LacZ cells (230±32 colonies/cm2 in TAC-2 int-3 cells versus 795±114 colonies/cm2 in TAC-2 LacZ cells), which suggests that Notch4(int-3) expression reduces plating (colony formation) efficiency in collagen gels. Accordingly, to avoid overestimating the inhibition of HGF-induced cord elongation in TAC-2 int-3 cells, the quantitative analysis of cord length and branching was carried out on a per colony basis, rather than on a per field basis (see Materials and

Methods). This analysis demonstrated that, despite the fact that colonies formed by TAC-2 int-3 cells are slightly more elongated and branched than those formed by TAC-2 LacZ cells, their morphogenic response to HGF is markedly decreased (Fig. 3C and 3D).

We analyzed the growth characteristics of the TAC-2 int-3 cell line, as described for TAC-2 Wnt-1 cells. TAC-2 int-3 cells plated on collagen coated dishes, either in the presence or absence of HGF, displayed no significant differences in cell number, morphology, or growth post-confluence when compared with TAC-2 controls (data not shown). Identical results were obtained with TAC-2 cell lines programmed to express Notch4(int-3) using a SV40 based retroviral vector (data not shown). Hence, the effects of Notch4(int-3) on TAC-2 branching morphogenesis are not correlated to changes in the growth properties in the cells.

The carboxy terminus of the Notch4(int-3) is not required for activity. The Notch4(int-3) oncoprotein, has most of the extracellular domain of Notch4 deleted and consists of the transmembrane and intracellular domains. To investigate which region(s) of Notch4(int-3) proteins are required and sufficient for activity, a series of Notch4(int-3) deletion mutants were generated (schematized in Fig. 5A). Four Notch4(int-3) deletion mutants were made and designated ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal domain) and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 5A). All four mutant int-2 cDNAs were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNCX. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of Notch4(int-3) deletion proteins of appropriate molecular weight in each respective cell line; this expression can be increased by sodium butyrate treatment (Fig. 5B). The ΔNTΔCT Notch4(int-3) deletion protein with predicted molecular weight of 25 kD co-migrates with a non specific anti-HA antibody

background band, but is clearly visible over background when these TAC-2 cells are treated with sodium butyrate (Fig. 5B). TAC-2 cell lines expressing the four different Notch4(int-3) deletion mutants were grown in collagen gels as described above, and the ability of each Notch4(int-3) deletion mutant to inhibit HGF induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 6, TAC-2 cells expressing either ΔNT (Fig. 6A and 6B), ΔCDC (Fig. 6C and 6D) or ΔNTΔCT (Fig. 6G and 6H) are responsive to HGF induced branching morphogenesis. In contrast, when grown in the presence of HGF, ΔCT expressing TAC-2 cells (Fig. 6E and 6F) display an identical phenotype as the TAC-2 int-3 cells. The carboxy terminus of the Notch4(int-3) is not required for Notch-mediated inhibition of TAC-2 branching morphogenesis. Thus, in this assay the activity of the Notch4(int-3) oncoprotein can be conferred by the amino terminus and cdc10 repeats.

Branching morphogenesis in cells co-expressing Wnt-1 and Notch4(int-3) oncoproteins. The activation of the Wnt-1 and Notch signaling pathways resulted in opposite effects on HGF- or TGF-β2-induced branching morphogenesis of TAC-2 cells. To explore the epistatic interactions between these two signaling pathways we investigated the effect of simultaneous expression of both Wnt-1 and Notch4(int-3) proteins on TAC-2 branching morphogenesis. The above described TAC-2 LacZ and TAC-2 int-3 cell lines, which were generated with the pLNCX expression vector, were now also programmed to express Wnt-1 using the retroviral vector pLHTCX. This vector drives gene expression from the CMV promoter and contains the hygromycin resistance gene. In this fashion, four additional TAC-2 cell lines were generated that were named TAC-2 LacZ/ctr, TAC-2 LacZ/Wnt-1, TAC-2 int-3/ctr and TAC-2 int-3/Wnt-1 (where ctr denotes control empty pLHTCX vector). To determine appropriate protein expression in each of these four cell lines, immunoblot analysis showed Notch4(int-3) and Wnt-1 proteins were expressed as expected and at levels similar to

those previously found to confer activity (data not shown). Each of the four cell lines were grown in collagen gels to determine their ability to undergo HGF- or TGF-β2-induced branching morphogenesis (Fig. 7), this assay was repeated three times with similar results. Doubly infected control cells TAC-2 LacZ/ctr (Fig. 7A, B, C) remained responsive to both HGF and TGF-β demonstrating that hygromycin and neomycin selection did not affect the phenotype of the TAC-2 cell lines. As observed previously for TAC-2 Wnt-1 cells, TAC-2 LacZ cells that are now programmed to express Wnt-1 (Fig. 7D, E, F) form small colonies that undergo modest branching even in the absence of HGF or TGF-β2; these cells form extensive elongated branches when grown in the presence of HGF or TGF-β2. The activity found for Notch4(int-3), that is the inhibition of HGF- and TGF-β-induced branching morphogenesis, was also found in the TAC-2 int-3/ctr cell line (Fig. 7G, H, I). Wnt-1 and Notch4(int-3) co-expressing cells, TAC-2 int-3/Wnt-1, are able to form colonies displaying branching and elongation and have an appearance similar to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J). An examination of several fields reveal that TAC-2 int-3/Wnt-1 cells displayed increased responses when treated with either HGF or TGF-β, thus these cells now regain responsiveness to these factors (Fig. 7K, L). Our results indicate that Notch activation attenuates responsiveness of TAC-2 cells to both HGF and TGF-β and that Wnt-1 can override the Notch activity in TAC-2 cells.

DISCUSSION

In this study, we have detailed a regulatory hierarchy involved in the branching morphogenesis of mammary epithelial cells. This regulation includes four distinct signaling pathways; the Wnt, Notch, HGF, and TGF-β signaling cascades. Using an in vitro model that reflects the branching morphogenesis exhibited during mammary gland development we have assessed the potential interactions between several different signaling pathways. This approach has allowed us to establish the epistatic relationships between these pathways. One remarkable feature of the regulation of branching morphogenesis we describe is its similarity to the regulatory pathways leading to morphogenic events during Drosophila development.

Wnt proteins as branching morphogens in the mammary gland. Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGF-β. Wnt-1 proteins induce moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGF-β2. The extent of Wnt-1 induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGF-β2. Our evidence suggests that Wnt-1 acts as a morphogen in this capacity. First, Wnt-1 induces a change in a morphogenic event, the formation of branched epithelial tubules or cords. Second, Wnt-1 does not appear on its own to alter the growth properties of the TAC-2 cells. Finally, Wnt-1 can induce branching in an environment where cysts typically form; that is, in hydrocortisone and cholera toxin treated cultures. Such cultures form spheroidal cysts enclosing a widely patent lumen. In this environment, peptide growth factors that display mitogenic and not morphogenic properties would increase the size of the cyst but the spheroidal structure would be maintained. In contrast, Wnt-1 alters the morphogenic behavior in such a way that new branch points are formed and the structures take on a tubular morphology.

Several Wnt proteins are expressed in the mammary gland during periods of morphological changes of the ductal epithelium (16, 50). Ectopic expression of Wnt-1 *in vivo* suggests a role for Wnt proteins in cell proliferation during mammary gland development; however, morphogenic changes also occur in response to Wnt-1. It is has been proposed that the Wnt-1 expression mimics the activity of an endogenous mammary gland Wnt proteins. A transgenic line driving expression of *Wnt*-1 to the mammary gland displays a hyperplastic phenotype, indicative of increased proliferation (48). In addition, both virgin females and males display a marked increase in the number of terminal branches, and in fact resemble the hormonally stimulated glands normally observed in pregnant animals. Tissue reconstitution experiments in which *Wnt*-1 is ectopically expressed in mammary epithelium also result in a hyperplastic gland where duct epithelium show abundant fine side-branches, suggesting that Wnt-1 may instruct the epithelium to form branches (13). This phenotype is most likely not simply a consequence of proliferation as it is not seen with a variety of other oncogenic proteins which when ectopically expressed in the mammary gland induce hyperplasia without increasing branching (12).

The Wnt signal transduction pathway is mediated in part through β-catenin, a protein associated with cadherins, and which is necessary for the adhesive functions of adherens junctions (29). Wnt-1 signaling results in stabilization of the cytoplasmic pool of β-catenin, which then can associate with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression (30, 31). TAC-2 cells programmed to express Wnt-1 in fact display increased levels of cytosolic β-catenin, when compared to TAC-2 LacZ cells (our unpublished data). This stabilization may be regulated by the phosphorylation of β-catenin on serine/threonine residues, possibly by glycogen synthase kinase 3 (GSK-3) (52). Recent evidence has demonstrated the importance of β-catenin/cadherin interactions in regulating cell adhesion, cell migration and epithelial phenotype in embryonic development (19). The activation of β-catenin

by Wnt-1 induced signaling may result in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells.

Cooperative interactions between Wnt-1 and HGF or TGF-β. In response to the combined effects of Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. We propose that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that Wnt signaling synergizes with the HGF/c-met tyrosine kinase pathway. The possibility that Wnt proteins cooperate in vivo with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the overlapping temporal patterns of *Wnt* genes and HGF/c-met expression (16, 36, 50).

One potential area where these two signaling pathways could converge might be through their effects on the catenin and cadherin proteins. The cooperation between Wnt-1 and HGF may be explained by their combined activation of β-catenin. β-catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF (22, 43). In addition, the Ras pathway is essential for the biological activity induced by HGF/c-met (20) and β-catenin has been demonstrated to be a substrate for tyrosine kinases and to become tyrosine phosphorylated in cells expressing activated Src and Ras (4). Another catenin-like protein, p120, which was identified as a substrate of Src and several receptor tyrosine kinases, interacts with the cadherin-β-catenin complex and may participate in regulating the adhesive function of cadherins (10). EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II and KGF can not (47). These activities correlate with the reported phosphorylation of β-catenin by the

EGF and HGF signal transduction pathways (43). It is yet unclear how tyrosine phosphorylation of β-catenin might regulate its activity. Tyrosine phosphorylated β-catenin is found in a detergent soluble pool (22, 26), which may reflect specific phosphorylation of a free pool of β-catenin. Since both Wnt-1 and HGF signaling can converge on β-catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined action on β-catenin activity.

Wnt-1, HGF, and TGF-β could induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell-substrate adhesion. TGF-β signaling involves receptors with serine/threonine kinase activity which are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGF-β could be mediated by a remodeling of extracellular matrix components and cell-substrate interactions. The Wnt signal transduction pathway may also regulate cell-substrate interactions, and the combined activity of both Wnt-1 and TGF-β may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

In *Drosophila*, wingless (*wg*) and the TGF-β homologue *Decapentaplegic* (*Dpp*) have been shown in some cases to act in combination to regulate gene transcription during inductive events. In particular, Wg and Dpp have been shown to act in combination during limb development (6, 11) and to induce *Ultrabithorax* expression during endoderm induction (39). Wnt-1 and TGF-β signaling may similarly converge to affect gene transcription during branching morphogenesis in the mouse mammary gland.

Notch inhibits branching morphogenesis of mammary epithelial cells. We demonstrate that Notch activation inhibits both the HGF and TGF-β induced branching morphogenesis of TAC-2 mammary epithelial cells. The precise mechanism of this inhibition is unclear. Activation of Notch signaling has been demonstrated to inhibit or alter the cell fate commitment or differentiation of a variety of different cell types (1, 18). For instance, *C. elegans* Lin-12 controls cell fate decisions during gonadogenesis, *Drosophila Notch* acts to control cell fate during neuroblast and photoreceptor cell differentiation, an activated *Xenopus Notch* can affect epidermal and neural crest cell development, and an activated mouse *Notch*1 can control cell fate during myogenesis and neurogenesis of cultured mouse cells. Transgenic mice that use the MMTV viral promoter to express the Notch4(int-3) oncoprotein, the activated form of Notch4, display severely impaired mammary ductal growth (25). When *Notch4(int-3)* is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited (15). These experiments demonstrate that *Notch4(int-3)*, like many other activated *Notch* genes, can act as a regulator of cell fate decisions in the mammary gland of mice. Little is known about the spatial and temporal pattern of *Notch* gene expression in the mammary gland, however, *Notch4* is expressed *in vivo* in the murine mammary gland (42, 45). *Notch* genes may thus regulate the cell fate decisions occurring during mammary gland development that lead to the branched epithelial strutcture of the gland.

We have found that Notch activation can affect the response of TAC-2 mammary epithelial cells to either HGF or TGF-β. Since HGF acts through a tyrosine kinase receptor and TGF-β acts through serine/threonine kinase receptors, the affects of Notch activation may involve more than specific inhibition of a particular signaling cascade. Notch may regulate the competency of TAC-2 cells to respond to several different factors, possibly by shifting TAC-2 cells to a fate that is not predisposed to undergoe

branching morphogenesis. This model would be consistent with the proposed activites of Notch proteins in several different organisms. Alternately, these signaling pathways may be controlled by Notch at a point at which they may converge to induce expression of genes important for branching morphogensis. Recently, the intracellular domain of LIN-12, a *C. elegans* Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH2 domain (24). This finding raises the possibility that the Notch signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the Notch4(int-3) oncoprotein is not required for biological activity. However, the amino terminal domain and the cdc10 repeats are required for Notch4(int-3) activity. These findings are consistent with previous observed data for other *Notch* genes. The RAM23 domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling (23). Deletion of the amino terminal domain of Notch4(int-3), which contains the RAM23 domain, may eliminate binding to CBF-1, and hence destroy Notch4(int-3) activity. The region of the LIN-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with another downstream and positive regulator, EMB5 (24). Point mutations and deletions within the cdc10 repeats result in loss of function of Notch proteins (17). Our data thus indicates that Notch4 might interact and be regulated through similar mechanisms.

Competing influences of Wnt and Notch signaling in branching morphogenesis. When TAC-2 cells are programmed to express both Wnt-1 and Notch4(int-3), the cells are able to undergo branching morphogenesis. In Wnt-1 and Notch4(int-3) coexpressing TAC-2 cells, branching morphogenesis can be increased by either HGF or TGF-β; that is, the cells regain responsiveness to these factors. The phenotype observed

in Wnt-1 and Notch4(int-3) coexpressing cells was similar to that of TAC-2 cells expressing only Wnt-1. The opposite biological activities of Wnt-1 and Notch4(int-3) observed in the TAC-2 cell assay correlate well with the mammary gland phenotype observed in Wnt-1 and Notch4(int-3) transgenic mice that ectopically express these proteins in the mammary gland (25). Although both oncogenes increase mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development whereas Notch4(int-3) inhibits ductal development.

Wnt-1 can override the Notch4(int-3)-mediated inhibition of branching morphogenesis providing the first evidence of interaction between these two signaling pathways in vertebrates. The dominance of Wnt-1 over activated Notch we have seen in murine cells parallels the functional relationship proposed for *Drosophila Wnt* (*wingless*) and *Notch* during *Drosophila* development (2). In this study, genetic analysis suggests a pathway convergence between wingless and Notch signaling resulting in opposing effects during patterning of the developing *Drosophila* wing. Activation of the wingless signal leads to regulation of Notch activity, possibly by *Drosophila* dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway. Analysis using a yeast interaction trap system suggested that Dishevelled may physically associate with the intracellular domain of Notch. The antagonism between Wnt-1 and Notch4(int-3) seen in branching morphogenesis may also be mediated by common regulators of the two signaling pathways such as Dishevelled.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal-epithelial and epithelial-epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative or differentiative response. Notch inhibition of ductal morphogenesis may be an early event in ductal morphogenesis. An attractive mechanism for overcoming Notch and allowing ductal morphogenesis to initiate or progress would be to activate the expression of a *Wnt* gene(s). Wnt could then serve

the dual function of suppressing Notch activity and initiating branching morphogenesis. Wnt signaling may then cooperate with other signaling pathways, such as those mediated by HGF and TGF-β, in order to complete branching morphogeneis. Our study thus has revealed complex interactions between the signal transduction pathways of Wnt, Notch, HGF and TGF-β, in regulating the branching morphogenesis of mammary epithelial cells.

ACKNOWLEDGMENTS

We thank Iva Greenwald, Martin Julius, John Krolewski, Ronald Liem, Stefan Pukatzki, Andrew Tomlinson, Cissy Young and Guangyu Wu for helpful discussions and critically reading the manuscript; Ronald Liem and James Goldman for assistance with microscopy.

This work was supported by a grant to J.K. from the US Army Medical Research and Material Command (USAMRMC) under grant DAMD17-94-J-4410 and the Marilyn Bokemeier Sperry Fund, by a pre-doctoral fellowship to H.U. from the USAMRMC under grant DAMD17-94-J-4153, and by a grant to R.M. from the Swiss National Science Foundation (no. 31-43364.95).

REFERENCES

1. **Artavanis-Tsakonas, S., K. Matsuno, and M. E. Fortini.** 1995. Notch signaling. Science **268**:225-32.

2. **Axelrod, J. D., K. Matsuno, S. Artavanis-Tsakonas, and N. Perrimon.** 1996. Interaction between Wingless and Notch signaling pathways mediated by dishevelled. Science **271**:1826-32.

3. **Bates, P., R. Fisher, A. Ward, L. Richardson, D. J. Hill, and C. F. Graham.** 1995. Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). Br. J. Cancer **72**:1189-93.

4. **Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M. M. Mareel, and W. Birchmeier.** 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-Src gene. J. Cell Biol. **120**:757-766.

5. **Buhler, T. A., T. C. Dale, C. Kieback, R. C. Humphreys, and J. M. Rosen.** 1993. Localization and quantification of wnt-2 gene expression in mouse mammary development. Dev. Biol. **155**:87-96.

6. **Campbell, G., T. Weaver, and A. Tomlinson.** 1993. Axis specification in the developing Drosophila appendage: The role of wingless, decapentaplegic, and the homeobox gene aristaless. Cell **74**:1113-1123.

7. **Coleman-Krnacik, S., and J. M. Rosen.** 1994. Differential temporal and spatial gene expression of fibroblast growth factor family members during mouse mammary gland development. Mol. Endocrinol. **8**:218-229.

8. **Daniel, C. W., S. Robinson, and G. B. Silberstein.** 1996. The Role of TGF-β in Patterning and Growth of the Mammary Ductal Tree. J. Mam. Gland Biol. Neoplasia **1**:331-341.

9. **Daniel, C. W., and G. B. Silberstein.** 1987. Postnatal Development of the Rodent Mammary Gland, p. 3-31. *In* M. C. Neville, and C. W. Daniel (ed.), The Mammary Gland. Plenum Press, New York and London.

10. **Daniel, J. M., and A. B. Reynolds.** 1995. The tyrosine kinase substrate p120cas binds directly to E-cadherin but not to the adenomatous polyposis coli protein or alpha-catenin. Mol. Cell. Biol. **15**:4819-24.

11. **Diaz-Benjumea, F. J., B. Cohen, and S. M. Cohen.** 1994. Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. Nature **372**:175-9.

12. **Edwards, P. A.** 1993. Tissue reconstitution models of breast cancer. Cancer Surveys **16**:79-96.

13. **Edwards, P. A. W., S. E. Hiby, J. Papkoff, and J. M. Bradbury.** 1992. Hyperplasia of mouse mammary epithelium induced by expression of the Wnt-1 (int-1) oncogene in reconstituted mammary gland. Oncogene **7**:2041-2051.

14. **Gallahan, D., and R. Callahan.** 1997. The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). Oncogene **14**:1883-90.

15. **Gallahan, D., C. Jhappan, G. Robinson, L. Hennighausen, R. Sharp, E. Kordon, R. Callahan, G. Merlino, and G. H. Smith.** 1996. Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res. **56**:1775-85.

16. **Gavin, B. J., and A. P. McMahon.** 1992. Differential regulation of the *Wnt*-gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. Mol. Cell. Biol. **12**:2418-2423.

17. **Greenwald, I.** 1994. Structure/function studies of lin-12/Notch proteins. Curr. Opin. Genet. Dev. **4**:556-62.

18. **Greenwald, I., and G. M. Rubin.** 1992. Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. Cell **68**:271-81.

19. **Gumbiner, B. M.** 1996. Cell Adhesion: The Molecular Basis of Tissue Architecture and Morphogenesis. Cell **84**:345-357.

20. **Hartmann, G., K. M. Weidner, H. Schwarz, and W. Birchmeier.** 1994. The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. J. Biol. Chem. **269**:21936-9.

21. **Haslam, S. Z., L. J. Counterman, and K. A. Nummy.** 1992. EGF receptor regulation in normal mouse mammary gland. J. Cell. Physiol. **152**:553-7.

22. **Hoschuetzky, H., H. Aberle, and R. Kemler.** 1994. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor. J. Cell. Biol. **127**:1375-1380.

23. **Hsieh, J. J., T. Henkel, P. Salmon, E. Robey, M. G. Peterson, and S. D. Hayward.** 1996. Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol. Cell. Biol. **16**:952-9.

24. **Hubbard, E. J., Q. Dong, and I. Greenwald.** 1996. Evidence for physical and functional association between EMB-5 and LIN-12 in Caenorhabditis elegans. Science **273**:112-5.

25. **Jhappan, C., D. Gallahan, C. Stahle, E. Chu, G. H. Smith, G. Merline, and R. Callahan.** 1992. Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev. **6**:345-355.

26. **Kinch, M. S., G. J. Clark, C. J. Der, and K. Burridge.** 1995. Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J. Cell Biol. **130**:461-71.

27. **Kordon, E. C., R. A. McKnight, C. Jhappan, L. Hennighausen, G. Merlino, and G. H. Smith.** 1995. Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. Dev. Biol. **168**:47-61.

28. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. Bio. Techniques 7:980-990.

29. **Miller, J. R., and R. T. Moon.** 1996. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev. **10**:2527-39.

30. **Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers.** 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell **86**:391-9.

31. **Morin, P. J., A. B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K. W. Kinzler.** 1997. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science **275**:1787-90.

32. **Niranjan, B., L. Buluwela, J. Yant, N. Perusinghe, A. Atherton, D. Phippard, T. Dale, B. Gusterson, and T. Kamalati.** 1995. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development **121**:2897-908.

33. **Nusse, R., A. van Ooyen, D. Cox, Y. K. Fung, and H. Varmus.** 1984. Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature **307**:131-136.

34. **Nusse, R., and H. E. Varmus.** 1992. *Wnt* genes. Cell **69**:1073-1087.

35. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90**:8392-8396.

36. **Pepper, M. S., J. V. Soriano, P. A. Menoud, A. P. Sappino, L. Orci, and R. Montesano.** 1995. Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp. Cell Res. **219**:204-10.

37. **Pierce, D. F., Jr., M. D. Johnson, Y. Matsui, S. D. Robinson, L. I. Gold, A. F. Purchio, C. W. Daniel, B. L. Hogan, and H. L. Moses.** 1993. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. Genes Dev. **7**:2308-17.

38. **Pitelka, D. R., S. T. Hamamoto, J. G. Duafala, and M. K. Nemanic.** 1973. Cell contacts in the mouse mammary gland. I. Normal gland in postnatal development and the secretory cycle. J. Cell Biol. **56**:797-818.

39. **Riese, J., X. Yu, A. Munnerlyn, S. Eresh, S. C. Hsu, R. Grosschedl, and M. Bienz.** 1997. LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. Cell **88**:777-87.

40. **Robbins, J., B. J. Blondel, D. Gallahan, and R. Callahan.** 1992. Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. J. Virol. **66**:2594-9.

41. **Russo, I. H., M. Tewari, and J. Russo.** 1989. Morphology and Development of the Rat Mammary Gland, p. 233-252. *In* T. C. Jones, V. Mohr, and R. D. Hunt (ed.), Integument and Mammary Glands. Springer-Verlag, Berlin.

42. **Sarkar, N. H., S. Haga, A. F. Lehner, W. Zhao, S. Imai, and K. Moriwaki.** 1994. Insertional mutation of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: normal- and tumor-tissue-specific expression of int-3 transcripts. Virology **203**:52-62.

43. **Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, N. Oku, K. Miyazawa, N. Kitamura, M. Takeichi, and F. Ito.** 1994. Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adhesion & Communication **1**:295-305.

44. **Smith, G. H.** 1996. TGF-β and Functional Differentiation. J. Mam. Gland Biol. Neoplasia **1**:343-352.

45. **Smith, G. H., D. Gallahan, F. Diella, C. Jhappan, G. Merlino, and R. Callahan.** 1995. Constitutive expression of a truncated INT-3 gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth & Differ. **6**:563-577.

46. **Soriano, J. V., L. Orci, and R. Montesano.** 1996. TGF-beta1 induces morphogenesis of branching cords by cloned mammary epithelial cells at subpicomolar concentrations. Bioch. Biophys. Res. Commun. **220**:879-85.

47. **Soriano, J. V., M. S. Pepper, T. Nakamura, L. Orci, and R. Montesano.** 1995. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. J. Cell Sci. **108**:413-30.

48. **Tsukamoto, A. S., R. Grosschedl, R. C. Guzman, T. Parslow, and H. E. Varmus.** 1988. Expression of the *int*-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell **55**:619-625.

49. **Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski.** 1996. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development **122**:2251-9.

50. **Weber-Hall, S. J., D. J. Phippard, C. C. Niemeyer, and T. C. Dale.** 1994. Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. Differentiation **57**:205-14.

51. **Yang, Y., E. Spitzer, D. Meyer, M. Sachs, C. Niemann, G. Hartmann, K. M. Weidner, C. Birchmeier, and W. Birchmeier.** 1995. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J. Cell Biol. **131**:215-26.

52. **Yost, C., M. Torres, J. R. Miller, E. Huang, D. Kimelman, and R. Moon.** 1996. The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev. **10**:1443-1454.

FIGURE LEGENDS

Figure 1

Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express either LacZ, Wnt-1 or int-3 were grown in the presence or absence of sodium butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

Figure 2

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A,B,C), Wnt-1HA (D,E,F), or int-3HA (G,H,I) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G), in the presence of HGF (B,E,H), or TGF-β2 (C,F,I). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGF-β2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (compare E to B, and to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGF-β2 (compare G to H or I, H to B, and I to C).

Figure 3

Wnt-1 and HGF have cooperative effects on branching morphogenesis of TAC-2 cells, while expression of int-3 inhibits HGF-induced branching morphogenesis. TAC-2 LacZ, TAC-2 Wnt-1 and TAC-2 int-3 cells were suspended in collagen gels at $5x10^3$ cells/ml (A and B) or $1x10^4$ cells/ml (C and D) and incubated with either control medium or 10ng/ml HGF for 7 days. In each of 3 separate experiments, at least three randomly

selected fields per condition were photographed. The total additive length of all cords in each field (A), the number of cord branch points per field (B), the total additive length of all cords in each individual colony (C), and the number of cord branch points per colony (D) was determined as described in Materials and Methods. Values are mean ± s.e.m.; n=3. Values for HGF are significantly ($P<0.001$) different when compared to controls (except for TAC-2 int-3 cells) and values are significantly different ($P<0.001$) when TAC-2 LacZ and TAC-2 Wnt-1 cell lines are compared. Similar results were obtained by evaluating cord length and branching per individual TAC-2 LacZ andTAC-2 Wnt-1 colony (data not shown).

Figure 4

Differential behavior of TAC-2 LacZ cells and TAC-2 Wnt-1 cells in hydrocortisone-supplemented cultures. Cells were suspended in collagen gels at $5x10^3$ cells/ml and incubated for 10 days with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin. Under these conditions, TAC-2 LacZ cells forn thick-walled spheroidal cysts enclosing a widely patent lumen (A), as previosly shown for untransfected cells. In marked contrast, TAC-2 Wnt-1 cells form short branched tubules (B). The three-dimensional structures illustrated in A and B are representative of the vast majority of colonies formed by TAC-2 LacZ and TAC-2 Wnt-1 cells, respectively. Magnification = 180x.

Figure 5

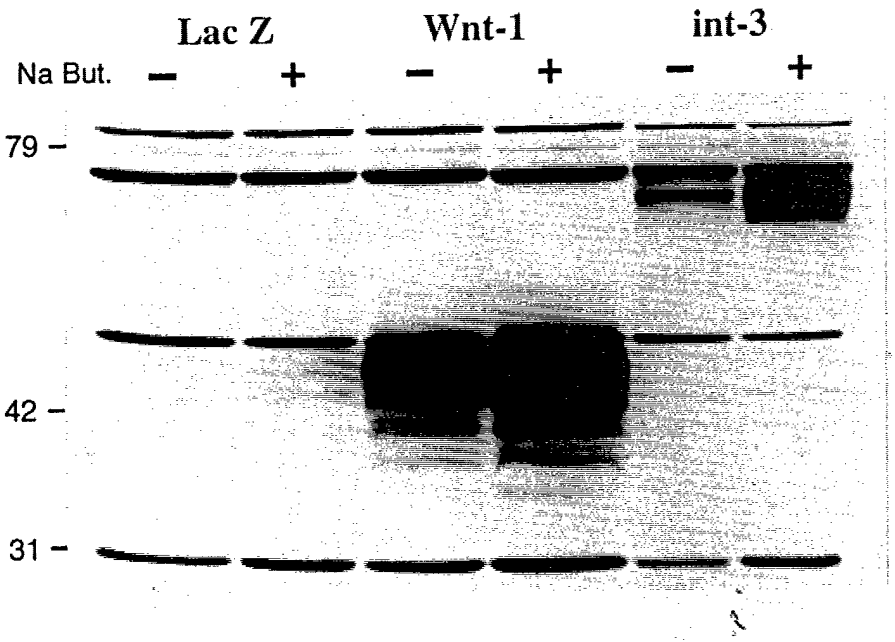
Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B). TAC-2 cells programmed to express either ΔNT, ΔCDC, ΔCT, and ΔNTΔCT were grown in the presence or absence of sodium butyrate. The int-3 deletion proteins are epitope tagged and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

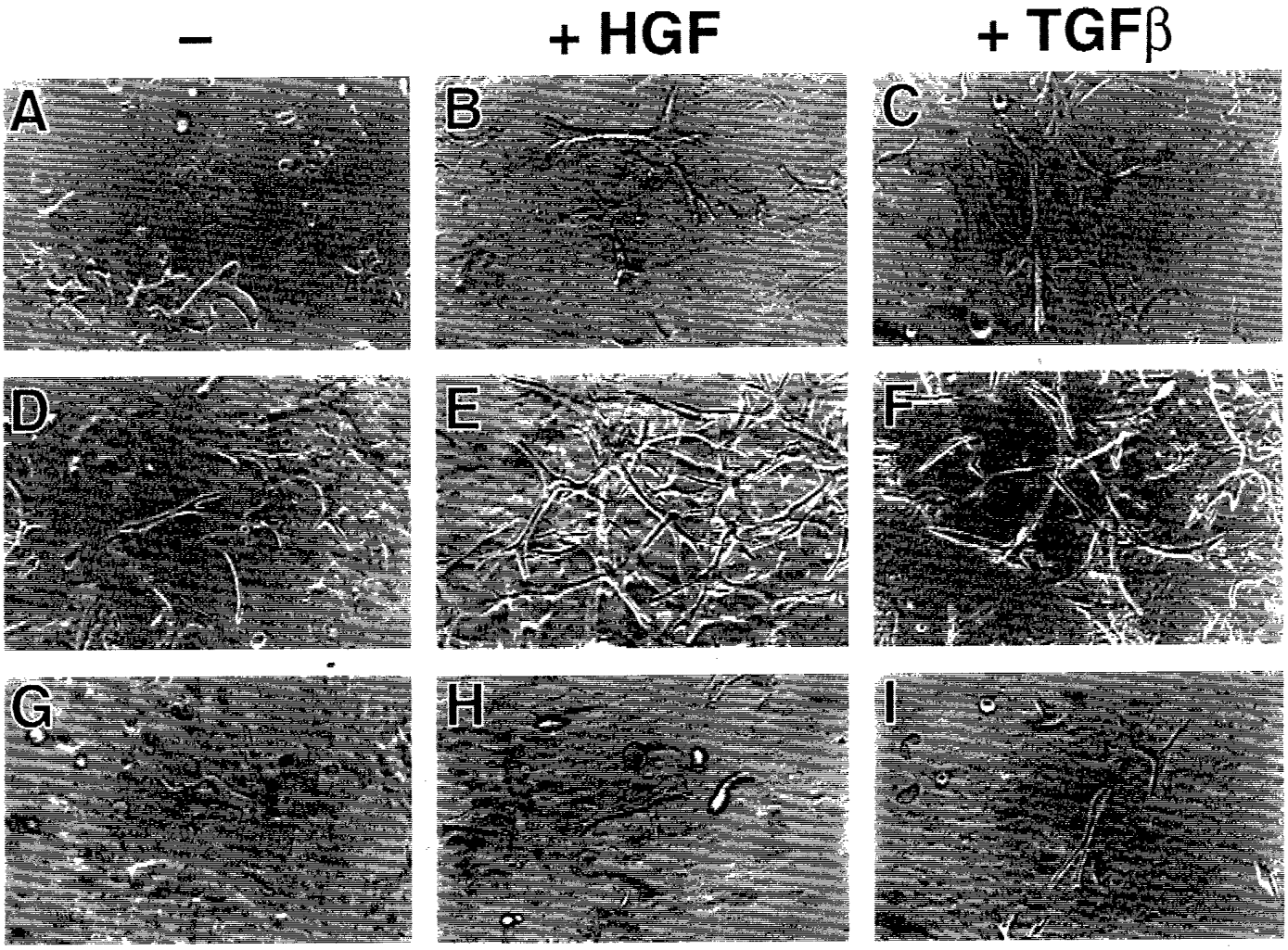
Figure 6

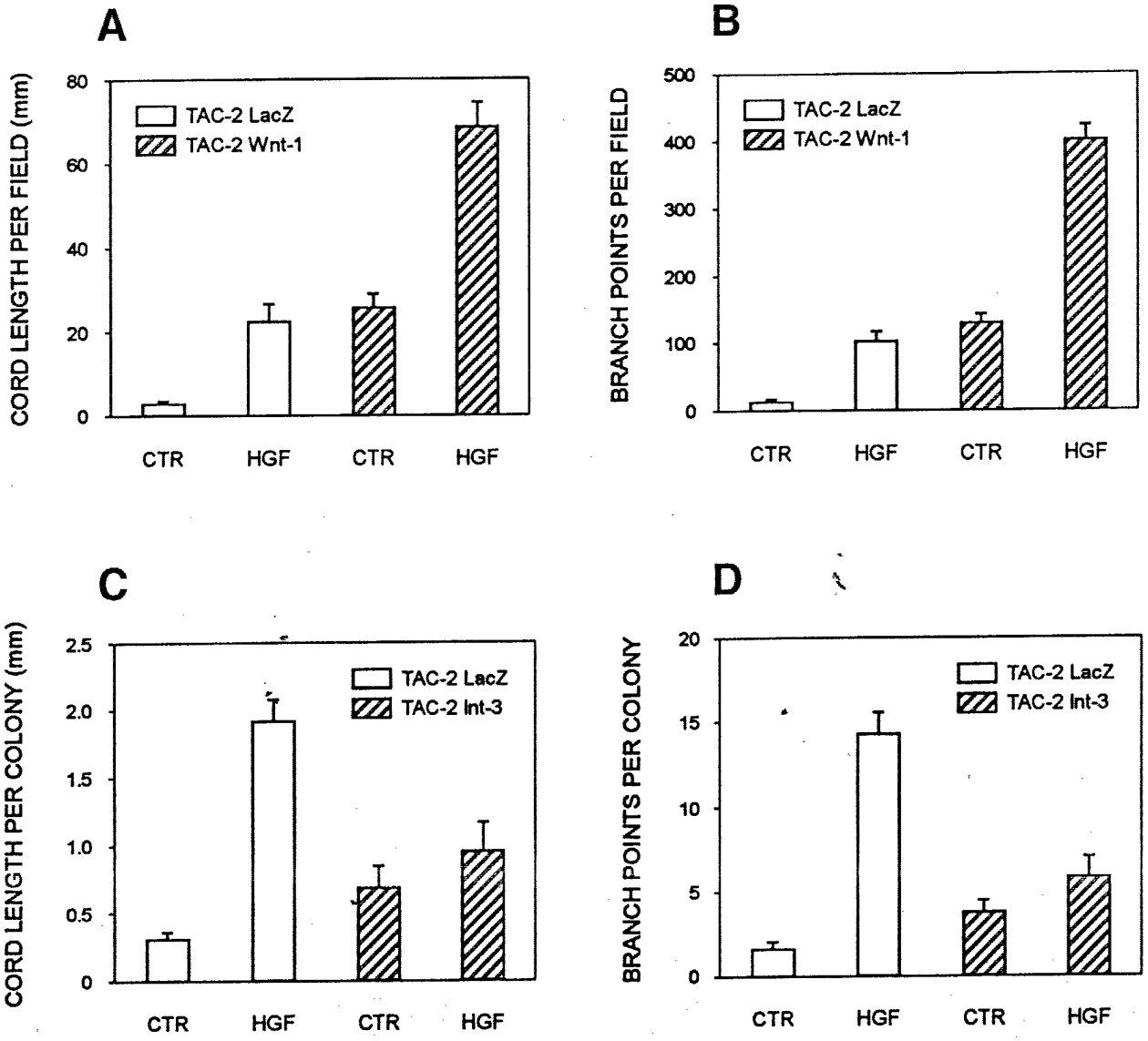
TAC-2 cell ductal morphogenesis assay with int-3 mutants. TAC-2 cells programmed to express ΔNT (A,B), ΔCDC (C,D), ΔCT (E,F), and ΔNTΔCT (G,H) were grown in collagen gels either in the absence of exogenous growth factor (A,C,E,G), or in the presence of HGF (B,D,F,H). HGF induces branching morphogenesis of TAC-2 ΔNT cells (B), TAC-2 ΔCDC cells (D) and TAC-2 ΔNTΔCT cells (H) TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of either HGF (F).

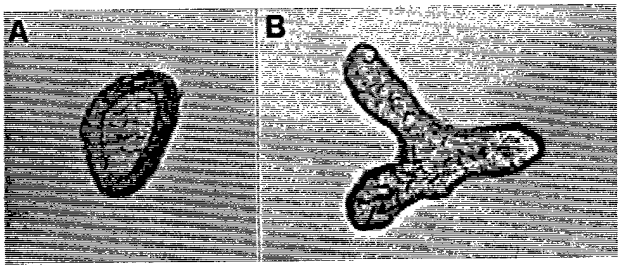
Figure 7

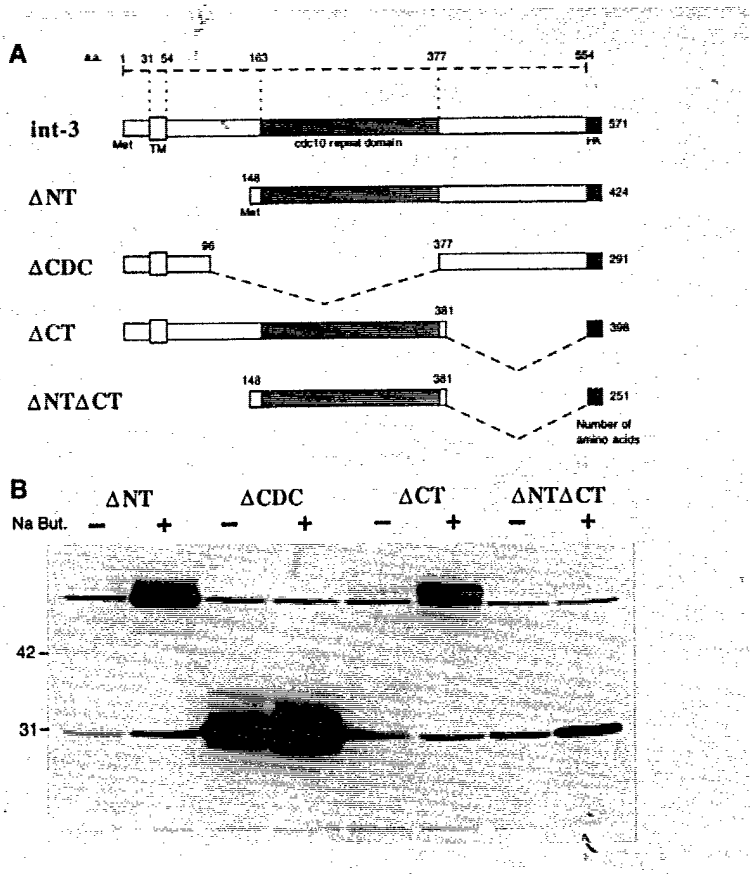
TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/ctr (A,B,C), LacZ/Wnt-1 (D,E,F), int-3/ctr (G,H,I), or int-3/Wnt-1 (J,K,L) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (20 ng/ml)(B,E,H,K), or in the presence of TGF-β2 (50 pg/ml)(C,F,I,L). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ/ctr cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGF-β2 (D), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF (E) or TGF-β2 (F). TAC-2 cells programmed to express int-3/ctr fail to undergo branching morphogenesis when grown in the presence of either HGF (H) or TGF-β2 (I). TAC-2 cells programmed to express both int-3 and Wnt-1 undergo branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF (K) or TGF-β2 (L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D,E,F).

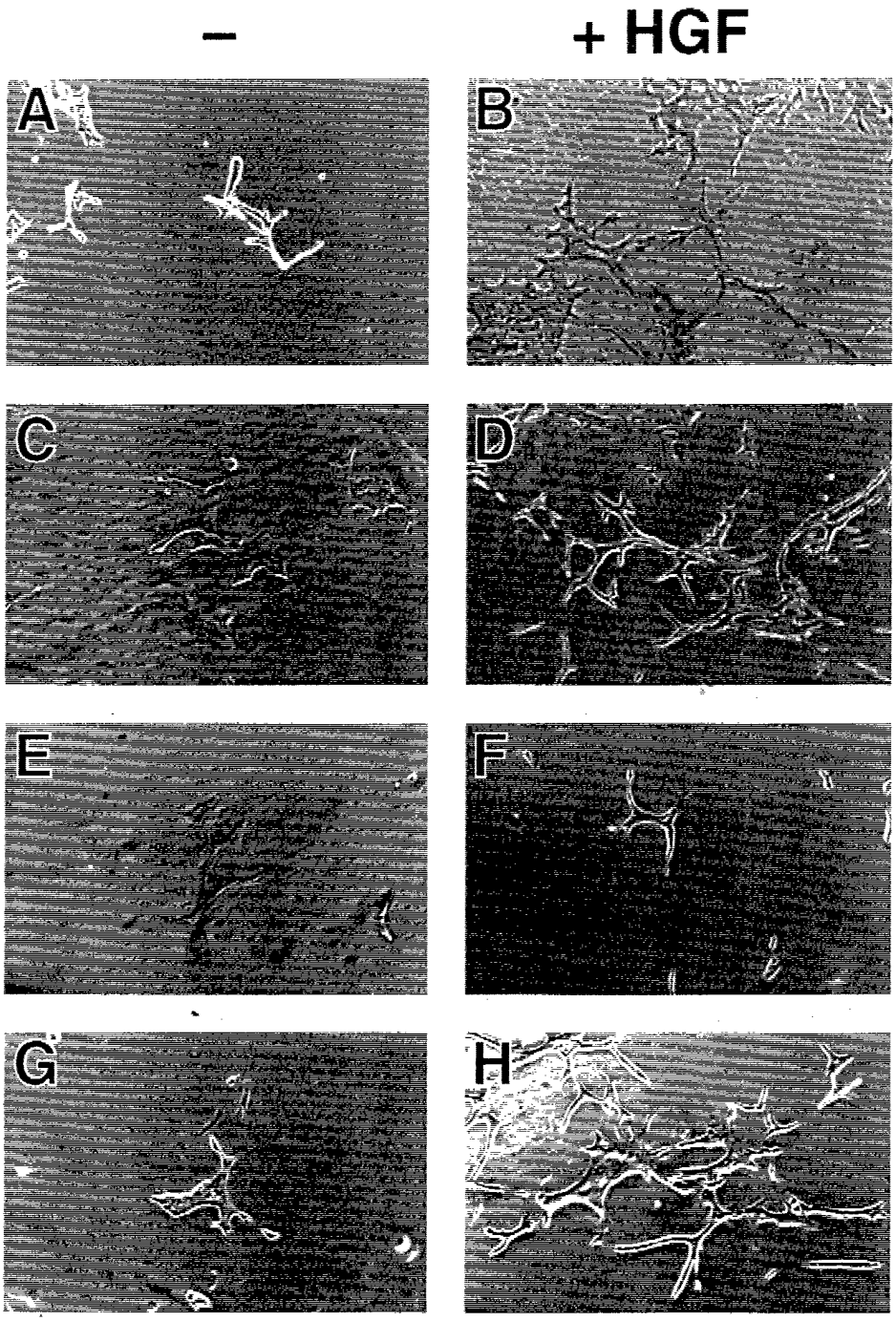

Lac Z
Wnt-1
int-3
Na But.
– + – + – +
79 –
42 –
31 –

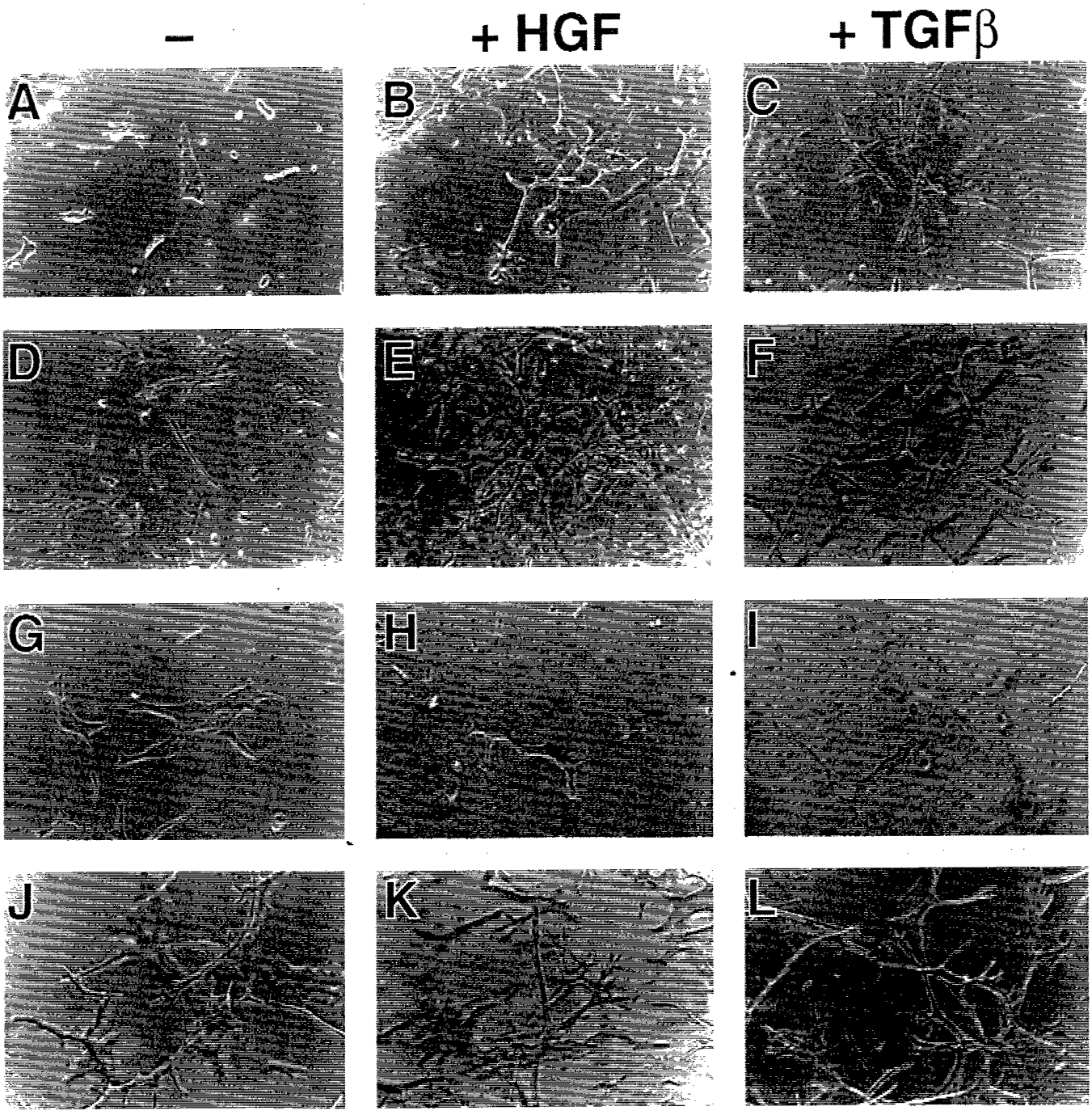

–
+ HGF
+ TGFβ
A
B
C
D
E
F
G
H
I


A
CORD LENGTH PER FIELD (mm)
80
60
40
20
0
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
B
BRANCH POINTS PER FIELD
500
400
300
200
100
0
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
C
CORD LENGTH PER COLONY (mm)
2.5
2.0
1.5
1.0
0.5
0
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF
D
BRANCH POINTS PER COLONY
20
15
10
5
0
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF


A
B


A
aa
1
31
54
163
377
554
Int-3
Met
TM
cdc10 repeat domain
PR
571
ΔNT
148
Met
424
ΔCDC
96
377
291
ΔCT
381
398
ΔNTΔCT
148
381
251
Number of amino acids
B
ΔNT
ΔCDC
ΔCT
ΔNTΔCT
Na But.
– + – + – + – +
42
31


–
+ HGF
A
B
C
D
E
F
G
H


–
+ HGF
+ TGFβ
A
B
C
D
E
F
G
H
I
J
K
L

sel-10, a negative regulator of *lin-12* activity in *C. elegans*, encodes a member of the CDC4 family of proteins

E. Jane Albert Hubbard*, Guangyu Wu+, Jan Kitajewski+, and Iva Greenwald*

* Dept. of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute

+ Dept. of Pathology and Center for Reproductive Sciences

Columbia University College of Physicians and Surgeons

New York, New York 10032

Running title: *sel-10*, a negative regulator of *lin-12*

corresponding author:

Iva Greenwald

Columbia University College of Physicians and Surgeons

701 West 168th Street Room 720

New York, New York 10032

telephone: (212) 305-6928/6931

FAX: (212) 305-1721

e-mail: greenwald@cuccfa.ccc.columbia.edu

Abstract

Mutations that influence *lin-12* activity in *C. elegans* may identify conserved factors that regulate the activity of *lin-12/Notch* proteins. We describe genetic evidence indicating that *sel-10* is a negative regulator of *lin-12/Notch* mediated signalling in *C. elegans*. Sequence analysis shows that SEL-10 is a member of the CDC4 family of proteins with a potential human ortholog. Co-immunoprecipitation data indicate that *C. elegans* SEL-10 complexes with LIN-12 and with murine Notch4. We propose that SEL-10 promotes the ubiquitin-mediated turnover of LIN-12/Notch proteins, and discuss potential roles for the regulation of *lin-12/Notch* activity by *sel-10* in cell fate decisions and tumorigenesis.

Introduction

Many cell-cell interactions that specify cell fate are mediated by receptors of the LIN-12/Notch family and ligands of the Delta/Serrate/LAG-2 (DSL) family (reviewed in Artavanis-Tsakonas et al., 1995). *C. elegans* affords an opportunity to study a simple case of lateral specification involving an interaction between two cells of the hermaphrodite gonad. These cells, named Z1.ppp and Z4.aaa, are initially equivalent in their developmental potential: each has an equal chance of becoming the anchor cell (AC), a terminally differentiated cell type that is necessary for vulval development, or a ventral uterine precursor cell (VU), which contributes descendants to the ventral uterus. However, in any given hermaphrodite, only one of these cells will become the AC, while the other becomes a VU (Kimble and Hirsh, 1979).

Laser ablation studies have shown that this process of lateral specification, the AC/VU decision, depends on interactions between Z1.ppp and Z4.aaa (Kimble, 1981; Seydoux and Greenwald, 1989). Furthermore, genetic studies have indicated that *lin-12*-mediated signalling controls the AC/VU decision: if *lin-12* activity is inappropriately elevated, Z1.ppp and Z4.aaa become VUs, while if *lin-12* activity is reduced, Z1.ppp and Z4.aaa become ACs (Greenwald et al., 1983). Genetic mosaic analysis (Seydoux and Greenwald, 1989) and reporter gene studies (Wilkinson et al., 1994) have indicated that both Z1.ppp and Z4.aaa initially express *lin-12* and

lag-2, but that a stochastic small variation in ligand and/or receptor activity is subsequently amplified by a feedback mechanism that influences *lin-12*and *lag-2* transcription. Thus, Z1.ppp and Z4.aaa assess their relative levels of *lin-12* activity as part of the decision-making process, before either cell commits to the AC or VU fates, and the feedback mechanism ensures that only one of the two cells will become an AC and the other will become a VU.

It is striking that the receptors (*lin-12/Notch* proteins), ligands (DSL proteins), and at least one downstream signalling component (CBF1/Su(H)/LAG-1; see Christensen et al., 1996 and references therein) that mediate lateral specification are highly conserved in animals as distantly related as *C. elegans*, Drosophila, and vertebrates. Furthermore, a feedback mechanism like that first described for the AC/VU decision (Seydoux and Greenwald, 1989) also exists for a Notch-mediated lateral interaction in Drosophila (Heitzler and Simpson, 1991) and seems likely to operate in Notch-mediated lateral interactions in vertebrates (e.g. Austin et al., 1995; Chitnis et al., 1995; Washburn et al., 1997). The identification of genes that influence *lin-12* activity during the AC/VU decision may reveal other conserved factors that participate in signal transduction or regulate the activity of *lin-12/Notch* proteins.

Genetic screens based on suppression or enhancement of *lin-12* mutations have identified a number of genes that influence *lin-12* activity. Here, we describe *sel-10*, which was first identified in a screen for suppressors of phenotypes associated with partial loss of *lin-12* activity (Sundaram and Greenwald, 1993). We have found that *sel-10* acts as a negative regulator of *lin-12* signalling, and that SEL-10 is a member of the CDC4 family of F-box/WD40 repeat containing proteins. CDC4, the most extensively studied member of this family, is a Saccharomyces cerevisiae protein that is involved in the ubiquitin-mediated degradation of cell cycle regulators such as SIC1 (reviewed in King et al., 1996). CDC4 binds to SIC1, thereby targeting the ubiquitination machinery to this substrate (R. Deshaies, personal communication). Similarly, we have shown that *C. elegans* SEL-10 can physically interact with the intracellular domains of *C. elegans* LIN-12 and murine Notch4 (Robbins et al., 1992; Uyttendaele et al., 1996). We propose

that SEL-10 promotes ubiquitin-mediated degradation of LIN-12/Notch proteins, and discuss potential roles for LIN-12/Notch turnover in cell fate decisions and oncogenesis.

Results

Lowering sel-10 *dosage elevates* lin-12 *activity*

Two *sel-10* alleles, *sel-10(ar28)* and *sel-10(ar41)*, were identified in a screen for suppressors of defects caused by a partial loss-of-function allele of *lin-12* (Sundaram and Greenwald, 1993). These *sel-10* alleles were shown to suppress multiple defects associated with loss of *lin-12* activity, and to enhance defects associated with elevated *lin-12* activity (Sundaram and Greenwald, 1993). Here, we provide evidence that *sel-10* alleles reduce *sel-10* activity, indicating that *sel-10* is a negative regulator of *lin-12* activity.

For the genetic analysis of *sel-10*, we relied on its genetic interactions with mutations in *lin-12*. We focused on two *lin-12*-mediated decisions (reviewed in Greenwald, 1997). One decision is made by two cells of the hermaphrodite gonad, Z1.ppp and Z4.aaa, between the anchor cell (AC) and ventral uterine precursor cell (VU) fates; normally, only one of these two cells becomes the AC, while the other becomes a VU (see Introduction). Eliminating *lin-12* activity causes both Z1.ppp and Z4.aaa to become ACs (the "2 AC defect"), and constitutively activating LIN-12 causes both Z1.ppp and Z4.aaa to become VUs. The other decision is made by the six vulval precursor cells, between a particular vulval fate termed "2°" or an alternative fate; normally, two of the six vulval precursor cells, P5.p and P7.p, adopt the 2° fate. Eliminating *lin-12* activity causes all six vulval precursor cells to adopt alternative non-2° fates, and constitutively activating LIN-12 causes all six vulval precursor cells to adopt the 2° fate. Thus, mutants in which LIN-12 is constitutively active display a "0 AC Egg-laying (Egl) defect" because the absence of an AC prevents normal vulval formation; they are also Multivulva (Muv), because the descendants of each vulval precursor cell that adopts the 2° fate forms a pseudovulva.

sel-10(ar41) appears to elevate *lin-12* activity: *sel-10(ar41)* suppresses the 2 AC defect of *lin-12* hypomorphs (Sundaram and Greenwald, 1993) and enhances the 0 AC defect caused by

elevated *lin-12* activity (compare Table 1, lines 1 and 2). Furthermore, the double mutant *lin-12(n379); sel-10(ar41)* displays a Muv phenotype characteristic of high *lin-12* activity that is not normally seen in *lin-12(n379)* single mutants (Table 1, lines 4 and 7).

The *sel-10* locus is haploinsufficient: we observed enhancement of the Muv defect (and the sterile/lethal defect) of *lin-12(n379)* hermaphrodites in *nDf42/+* hermaphrodites (Table 1, lines 4 and 6).

The *sel-10(ar41)* mutation appears to reduce *sel-10* activity: the enhancement of the Muv defect of *lin-12(n379)/+* hermaphrodites is more pronounced when *sel-10(ar41)* is placed in trans to the large deficiency *nDf42* (Table 1, lines 2 and 3). The greater enhancement seen in trans to a deficiency may mean that the *sel-10(ar41)* allele is a partial loss of function allele rather than a null allele; alternatively, *nDf42* may remove another gene that interacts with or is functionally redundant with *sel-10*. Molecular data (see below) indicate that *sel-10(ar41)* would lead to a drastic truncation of the predicted SEL-10 protein, suggesting that *sel-10(ar41)* strongly reduces *sel-10* activity.

Elevating sel-10 *dosage lowers* lin-12 *activity*

The molecular cloning of *sel-10(+)* (see below) enabled us to examine the effect of elevated *sel-10(+)* activity, since in general extrachromosomal arrays formed after injecting DNA at a high concentration result in higher transgene expression (Mello et al., 1991). We found that extrachromosomal arrays containing high-copy arrays of the *sel-10* genomic region (see below) appear to lower *lin-12* activity as assayed by their effect on the AC/VU decision. There is a dramatic decrease in the proportion of *lin-12(n379)* hermaphrodites displaying the 0 AC defect in the presence of the high copy number array *arEx93* (Table 2A). In addition, the presence of the *arEx93* array enhances the 2AC defect caused by a partial loss of *lin-12* function (Table 2B). Therefore, the level of *sel-10* activity can control the level of *lin-12* activity, since increasing or decreasing the activity of *sel-10* has reciprocal effects on *lin-12* activity.

sel-10 *mutants display low penetrance defects associated with constitutive activation of* lin-12

Most *sel-10* animals appear wild-type. We have observed that about 1% of *sel-10(ar41)* hermaphrodites lack an AC (data not shown). Furthermore, about 4% of *sel-10(ar41)* males display a gonad Migration (Mig) defect similar to that seen in *lin-12(d)* mutants, where it results from to failure to form the linker cell, the male counterpart of the hermaphrodite AC (Greenwald et al., 1983). In addition, we note that about 8% of *sel-10* mutant hermaphrodites are Egl even though they have an AC, and that *sel-10* males have a reduced mating efficiency that can not be completely accounted for by the Mig defect. These additional defects may reflect the effect of increased *lin-12* activity on other cell fate decisions (Greenwald et al., 1983).

Cell autonomy of sel-10 *effect on* lin-12 *activity*

Two lines of evidence suggest that *sel-10* functions cell autonomously to elevate *lin-12* activity. First, we examined the effect of reducing *sel-10* activity on the activity of the intracellular domain of LIN-12. Expression of *lin-12(intra)* causes phenotypes associated with LIN-12 activation (Struhl et al., 1993). Since LIN-12(intra) lacks the extracellular domain and hence is active in the absence of external signalling, an enhancement of *lin-12(intra)* activity by *sel-10* mutations would be evidence for cell autonomy of *sel-10* effect on *lin-12* activity. We used an extrachromosomal array that contains the *lin-12(intra)* transgene and a transformation marker (see Materials and Methods); this array results in a low-penetrance *lin-12* activated phenotype (Table 3, line 1). When this array is combined with *sel-10(ar41)*, there is a dramatic increase in the proportion of hermaphrodites displaying the 0 AC-Egl defect and males displaying the Mig defect (Table 3, line 2), suggesting that *sel-10(+)* activity normally reduces *lin-12* function in the same cell.

We have also tested whether *sel-10* functions in the receiving end of *lin-12*-mediated cell-cell interactions by performing cell ablation experiments to remove the signalling cell, in this case Z4.aaa (Table 3b). This experiment enables different genotypes to be compared with respect to their intrinsic level of constitutive *lin-12* activity in Z1.ppp. If Z4, the precursor to Z4.aaa, is ablated in *lin-12(n379)/+* hermaphrodites, Z1.ppp usually becomes an AC, because the level of

constitutive *lin-12* activity is relatively low. However, if Z4 is ablated in *lin-12(n379)/+; sel-10* hermaphrodites, Z1.ppp usually becomes a VU, suggesting that the level of constitutive *lin-12* activity is relatively high. These results suggest that *sel-10(+)* functions to reduce *lin-12* activity within the same cell, since a high level of intrinsic *lin-12* activity is seen when *sel-10* activity is reduced even when the signalling cell is removed.

Cloning of sel-10 *by an anti-suppression assay*

sel-10 was previously mapped to an interval between *lin-25* and *unc-76* on LGV (Sundaram and Greenwald, 1993). We refined the map position to a 300 kb interval between the cloned polymorphisms *arP3* and *TCPAR1* (see Materials and Methods and Fig. 1). Cosmids from the region were tested for their ability to reverse the suppression of the 2AC defect of *lin-12(ar170)* by *sel-10(ar41)* (see Materials and Methods). Arrays containing the cosmid C07E11 gave rescue in this anti-suppression assay and also reversed the enhancement of *lin-12(n379)* by *sel-10(ar41)* (data not shown). This cosmid was further subcloned and the ~8kb fragment in pJH166 gave results similar to that seen with the entire cosmid (Figure 1).

Molecular analysis of sel-10

The ends of pJH166 (Figure 1) were sequenced and compared with sequence generated by the *C.elegans* genome sequencing project (Waterston et al., 1997). The entire region was found on the cosmid F55B12. A fragment from the predicted open reading frame was radiolabeled and used to screen a Northern blot and to probe a cDNA library (see Materials and Methods). Northern analysis revealed a single band of ~2.5kb which is present in total RNA prepared from wild type, *sel-10(ar41)* and *sel-10(ar28)* strains (data not shown). The ends of ten cDNAs from the region were sequenced and the largest cDNA was sequenced in its entirety on one strand. Verification of the 5' end was obtained by sequencing products amplified from the cDNA library using the trans spliced leader sequence SL1 (Krause and Hirsh, 1987) and a *sel-10*-specific sequence for primers.

The splice junction of SL1 to the *sel-10* coding region occurs four bases upstream of the first predicted start codon. Fig. 2 summarizes the results of the sequence analysis of *sel-10*.

SEL-10 encodes a protein of the CDC4 family

A BLAST search (Altschul et al., 1990) using the predicted SEL-10 protein sequence revealed that it contains two previously identified amino acid sequence motifs (Fig. 3A-C). First, there is a motif (Kumar and Paietta, 1995) that is now called the F-Box, after its occurrence in cyclin F (Bai et al. 1996). The F-Box motif has been implicated in protein-protein interactions, and is found in a large variety of proteins, many of which contain other recognizable motifs C-terminal to the F-Box (Bai et al., 1996). Second, there are seven tandem WD40 repeats, also known as ßtransducin repeats, a conserved repeat of approximately 40 amino acids named for the common appearance of Trp-Asp (WD) at the end of the repeat (reviewed in Neer et al., 1994). The crystal structure of ßtransducin reveals that the seven repeats form a ß propeller structure, which most likely mediates protein-protein interactions (Gaudet et al., 1996; Lambright et al., 1996; Sondek et al., 1996). There is a great deal of functional diversity among WD40 repeat-containing proteins.

The presence of an F-box N-terminal to a set of seven WD40 motifs is the hallmark of the CDC4 family of WD40 repeat-containing proteins, indicating that SEL-10 belongs to this family. Furthermore, separate BLAST searches with just the SEL-10 F box or the SEL-10 WD40 repeats identified members of the CDC4 family as the most similar. The F-Box motif present in proteins within the CDC4 subfamily is more conserved than among other F-Box-containing proteins (Figure 3B), and there is more extensive homology around the F-box (Kumar and Paietta, 1995). In addition, the alignment of the WD40 repeats of SEL-10 and CDC4 (Fig. 3C) reveals that a given WD40 repeat is more similar between yeast and worms than are the repeats within a given species.

The CDC4 family includes proteins in fungi and vertebrates, other predicted *C. elegans* proteins (E. Kipreos, S. Gohel and E. Hedgecock, personal communication; E.J.A.H., unpublished observations), and several mammalian proteins (see Kumar and Paietta, 1995; Bai et

al., 1996). The best studied member of this family, *S. cerevisiae* CDC4, targets Sic1 and certain G1 cyclins for degradation (reviewed in King et al., 1996). However, not all CDC4 family members are cell cycle regulators. For example, there are proteins that negatively regulate sulfur metabolism from *S. cerevisiae*, *Neurospora crassa* and *Aspergillus nidulans* (Natorff et al., 1993; Kumar and Paietta, 1995; Thomas et al., 1995).

A recent database search has revealed potential human and rat SEL-10 orthologs (Fig. 3 D). The limited sequence information available to date for the human gene begins in the sixth WD40 repeat and extends to the C terminus, and in this region, there is 60% sequence identity. BLAST searches of the available databases using the SEL-10 sequence C-terminal to the WD40 repeats has not identified any other highly conserved sequences.

sel-10 *mutations truncate the SEL-10 protein*

Sequence analysis of *sel-10* mutations supports the genetic evidence suggesting that they strongly reduce *sel-10* activity. The sequence alterations caused by *sel-10(ar41)* and *sel-10(ar28)* were determined by direct sequencing of amplified genomic DNA products (see Materials and Methods). Both alleles are nonsense mutations at nucleotide positions 969 and 1533, respectively (see Fig. 2), resulting in truncated predicted proteins.

sel-10(ar41) removes the C terminal half of the protein, including five of the seven WD40 repeats. This observation suggests that *sel-10(ar41)* is likely to result in a nonfunctional SEL-10 protein. It is unlikely that the two WD40 repeats that remain in this protein are functional since there are no known WD40-repeat containing proteins with only two repeats (Neer et al.,1994). Furthermore, the crystal structure of ßtransducin reveals that the seven repeats form a ß propeller structure that would not be complete in the absence of five of the seven repeats (Sondek et al., 1996). Finally, comparable mutations in another *C. elegans* CDC4 subfamily protein, LIN-23, behave like molecular null alleles (E.T. Kipreos, S.P. Gohel, and E.M. Hedgecock, personal communication).

C. elegans SEL-10 physically interacts with LIN-12(intra) and murine Notch4(int3)

We probed for potential interactions between SEL-10 and the intracellular domains of LIN-12/Notch proteins, specifically LIN-12(intra), the intact intracellular domain (see Struhl et al., 1993) and Notch4(int3), the intact intracellular domain with some additional sequences produced by the *int3* mutation (Robbins et al., 1992; Uyttendaele et al., 1996). We initially used the yeast two-hybrid system (Fields and Song, 1989) and our preliminary results suggested that SEL-10 physically interacted with the *C. elegans* LIN-12 intracellular domain, the *C. elegans* GLP-1 intracellular domain (GLP-1 is another *C. elegans* LIN-12/Notch protein; see Yochem and Greenwald, 1989), and the mouse Notch4(int3) intracellular domain (data not shown).

To examine further whether SEL-10 binds LIN-12/Notch proteins, we carried out co-immunoprecipitation experiments using transfected mammalian cells (Fig. 4). 293T (Bosc23) cells (human embryonic kidney cells) were transiently transfected with hemagglutinin (HA)-tagged LIN-12(intra) and/or myc-tagged SEL-10 (see Materials and Methods). Transfected cells were lysed and LIN-12(intra)HA was precipitated with anti-HA antibodies (Fig. 4A) or, alternatively, SEL-10myc was immunoprecipitated with anti-myc antibodies (Fig. 4B). The immunoprecipitates were subjected to immunoblot analysis to identify bound proteins, and probed with anti-myc or anti-HA antibodies as indicated. Under both conditions, the immunoprecipitates were found to contain both LIN-12HA and SEL-10myc. This result suggests that SEL-10 and LIN-12 are able to interact physically, either directly or in a complex.

We also examined whether the *C. elegans* SEL-10 protein would interact with the murine Notch4(int-3) protein. Cells were transfected with Notch4(int-3) and/or SEL-10HA, and immunoprecipitation was performed with cell lysates using either anti-Notch4 antibodies (Fig 4C) or anti-HA antibodies (Fig. 4D). Immunoblot analysis demonstrated that the immunoprecipitates contained a complex of Notch4(int-3) and SEL-10 proteins; thus, SEL-10 is able to complex with Notch4(int-3) protein.

The observations that *sel-10* negatively regulates *lin-12* activity, that SEL-10 resembles CDC4, and that SEL-10 physically interacts with LIN-12 taken together strongly suggest that SEL-

10 functions biochemically like CDC4 to promote LIN-12 turnover. We have attempted to examine the effect of co-expressing *C. elegans* SEL-10 on the steady state levels and ubiquitination of Notch4(int-3) and observed a modest decrease in the steady state level (data not shown); however, at this time the mechanism underlying this decrease is not clear. Furthermore, there appears to be polyubiquitination of Notch4(int-3) even in the absence of transfected SEL-10, perhaps due to the activity of an endogenous mammalian *sel-10*-like gene.

Discussion

In this paper, we have presented genetic evidence indicating that *sel-10* is a negative regulator of *lin-12* mediated signalling in *C. elegans*. Mutations that lower *sel-10* activity elevate *lin-12* activity, and increasing *sel-10* dosage lowers *lin-12* activity, suggesting that the level of *sel-10* activity can influence *lin-12* activity. Furthermore, *sel-10* appears to act in the same cell as *lin-12*.

We propose that the mechanism by which *sel-10* affects *lin-12* activity may be by controlling LIN-12/Notch protein levels. Sequence analysis indicates that SEL-10 is related to the Saccharomyces cerevisiae protein CDC4, which promotes the ubiquitin-dependent degradation of cell cycle regulators (reviewed in King et al., 1996). CDC4 complexes with its substrates (R. Deshaies, personal communication), and we have found that *C. elegans* SEL-10 complexes with the intracellular domain of LIN-12. Proteins related to SEL-10 exist in mammals, and *C. elegans* SEL-10 physically complexes with Notch4(int3), the intracellular domain of murine Notch4. These observations suggest that negative regulation of LIN-12/Notch by SEL-10 may be an evolutionarily conserved feature.

SEL-10 may target LIN-12/Notch proteins for ubiquitin-mediated degradation

The attachment of ubiquitin to substrates involves a series of protein complexes. Ubiquitin is activated by linkage to an E1 ubiquitin activating enzyme, then transferred to an E2 ubiquitin conjugating enzyme. Some ubiquitination events also require the action of a third complex, termed E3. It is thought that E3 complexes may contribute to substrate specificity (reviewed in

Ciechanover, 1994; King et al., 1996). The Saccharomyces cerevisiae protein Cdc4p may function in an E3 complex. *CDC4* is one of a group of genes that also includes *CDC34*, *CDC53*, and *SKP1*; together, the proteins encoded by these genes directly regulate the level of the cyclin dependent kinase inhibitor SIC1, which must be destroyed for progression from G1 to S phase. CDC34 is an E2 ubiquitin conjugating enzyme (Goebl et al., 1988), and the current view is that CDC4, CDC53, and SKP1 function as an E3 complex (see Bai et al., 1996; Mathias et al., 1996). Based on our analysis of *sel-10* and the data for *CDC4*, we propose that SEL-10 functions as part of an E3 complex to target the intracellular domains of LIN-12/Notch proteins for ubiquitin-dependent degradation.

The carboxy terminal region of all LIN-12/Notch proteins contain PEST sequences and one or more lysines immediately C-terminal to the cdc10/SWI6 motifs. The presence of PEST sequences often indicates that ubiquitin-mediated turnover occurs, although the two are not necessarily strictly correlated (Rogers et al., 1986). With respect to the CDC34/CDC4-mediated events, it appears that the PEST sequences couple phosphorylation of the substrate to attachment of ubiquitin to lysine residues (reviewed in King et al., 1996). However, the PEST sequence found in the carboxy terminal region of all LIN-12/Notch proteins may not be required for SEL-10-mediated degradation of LIN-12/Notch proteins, because *sel-10* mutations can still enhance the gain-of-function phenotype of a *glp-1* allele (Mango et al., 1991) that is truncated prior to the PEST sequence (E.J.A.H., unpublished observations).

An important issue to consider in the context of SEL-10 as a component of an E3 complex is its specificity for LIN-12/Notch proteins. The available *C. elegans* genetic data suggest that *sel-10* is an allele-nonspecific, gene-specific suppressor of *lin-12*, supporting a role for SEL-10 specifically in regulating the activity of LIN-12, or perhaps a small set of proteins including LIN-12. Allele-nonspecificity is indicated by the observation that mutations in *sel-10* suppress/enhance all *lin-12* alleles tested (Sundaram and Greenwald, 1993; this work; E.J.A.H., unpublished observations). Gene-specificity is suggested by the fact that mutations in *sel-10* have not been identified in numerous screens in many laboratories for suppressors of hypomorphic

mutations in genes encoding proteins other than LIN-12/Notch proteins; furthermore, we have not observed suppression of various marker mutations used in routine strain constructions or of hypomorphic alleles of several other genes encoding receptor proteins (E.J.A.H., unpublished observations).

The available genetic data also suggest that *sel-10* activity does not regulate cell cycle progression, a possibility raised by the phenotype of *cdc4* mutants. Mutations in *cul-1*, a *C. elegans* gene related to S. cerevisiae *CDC53*, cause hyperplasia of larval blast cells, suggesting that *cul-1* regulates cell-cycle progression (see Kipreos et al., 1996). We have not seen any evidence that hyperplasia occurs in *sel-10(ar41)* mutants (E.J.A.H., unpublished observations). Since *sel-10(ar41)* mutants have little or no *sel-10* activity (see Results), we think that it is unlikely that *sel-10* is involved in cell cycle regulation per se, unless there is another functionally redundant gene that masks cell cycle involvement of *sel-10*. In contrast, mutations in another *CDC4* related gene, *lin-23*, do cause hyperplasia, consistent with a role for *lin-23* in the regulation of cell cycle progression (E.T. Kipreos, S.P. Gohel, and E.M. Hedgecock, personal communication).

Potential roles for LIN-12/Notch protein downregulation or turnover in cell fate decisions

Proteolysis of LIN-12/Notch proteins might occur in response to ligand binding or occur constitutively. For a variety of cell surface receptors, ligand-induced polyubiquitination appears to be a mechanism for downregulation (reviewed in Ciechanover and Schwartz, 1994). Although we do not at this time have direct evidence for ligand-induced ubiquitination of LIN-12/Notch receptors, we note that LIN-12(intra), which genetically and physically interacts with SEL-10, behaves like an activated receptor (Struhl et al., 1993). Alternatively, SEL-10 may target any form of LIN-12/Notch (activated or unactivated) for degradation. Although constitutive turnover is not strictly a mechanism for controlling receptor activity per se, it would in effect sensitize the system to other control mechanisms such as transcriptional regulation (see below) by generally reducing the amount of LIN-12.

Constitutive turnover or ligand-induced downregulation of LIN-12/Notch proteins may be important for cell fate decisions to occur normally. We can best illustrate potential roles for turnover or downregulation using the AC/VU decision (Seydoux and Greenwald, 1989; Wilkinson et al., 1994) as an example. Initially, Z1.ppp and Z4.aaa have equal signalling and receiving potentials; ligand (LAG-2) and receptor (LIN-12) may interact, but signalling activity is below a critical threshold. SEL-10-mediated turnover or downregulation of LIN-12 might prevent this initial signalling from causing both cells to achieve the threshold value of effector activity. Thus, one possible role for receptor turnover or downregulation would be to limit the output from a single ligand-receptor interaction.

Another potential role for receptor turnover or downregulation is in enhancing differences in *lin-12* activity between interacting cells. During the AC/VU decision, a small stochastic difference between the two cells is amplified by a feedback mechanism (Seydoux and Greenwald, 1989). The feedback mechanism appears to involve differential transcription of ligand and receptor genes: activation of LIN-12 appears to repress transcription of *lag-2* and to stimulate transcription of *lin-12* (Wilkinson et al., 1994). The feedback mechanism ensures that the cell with higher *lin-12* activity becomes the VU while the cell with lower *lin-12* activity becomes the AC. Downregulation of LIN-12 would be necessary for differences in transcription to be manifest. In the absence of downregulation, signalling from activated receptor would persist, masking the effects of differential transcription. Indeed, this situation is analogous to the role of ubiquitin-mediated degradation of G1 cyclins (see King et al., 1996).

Turnover of LIN-12/Notch proteins may play different or additional roles in other cell fate decisions. For example, in Drosophila eye development, Notch appears to be utilized for sequential cell fate decisions (Cagan and Ready, 1989), which would seem to necessitate clearance of activated Notch after each decision so that a new assessment of *Notch* activity can be made. Furthermore, it is also conceivable that for some LIN-12/Notch mediated decisions, the cell fate adopted may depend on the intensity of signal, as has been seen for receptors for gradient morphogens (e.g., Nellen et al., 1996). If any LIN-12/Notch-mediated decisions do display

such dosage sensitivity, it is likely that they would depend on rapid turnover of activated receptor complexes so that the correct threshold value is read.

The fact that most *sel-10(ar41)* individuals are phenotypically wild-type, with only a small proportion displaying phenotypes associated with LIN-12 activation, may be explained in this context if there is a redundant gene product or regulatory mechanism. There are other CDC4 related genes in the *C. elegans* genome (E.J.A.H., unpublished observations). Furthermore, there may be other mechanisms for degrading LIN-12. For example, *sel-1*, another negative regulator of *lin-12* activity, may also be involved in LIN-12 turnover (see Grant and Greenwald, 1997), but since SEL-1 is an extracytosolic protein, it is not likely to be directly involved in the ubiquitination of the intracellular domain of LIN-12.

Potential roles for sel-10 *in oncogenesis*

Mammalian tumors induced by expression of Notch4(int3) or other truncated forms of Notch largely consisting of the intact intracellular domain are thought to result from constitutive Notch activity (Ellisen et al., 1991; Robbins et al., 1992; Uyttendaele et al., 1996). Since SEL-10 downregulates Notch activity, it may act to restrain either normal or oncogenic functions of activated Notch, and hence suppress cell growth. If so, loss-of-function mutations in vertebrate *sel-10* could contribute to oncogenesis mediated by Notch by elevating the level of Notch protein. For instance, human T acute lymphoblastic leukemias, which in the majority of cases do not contain oncogenic Notch alterations (Drexler et al., 1995), and human breast tumors, which thus far have not been reported to contain oncogenic Notch alterations, may carry mutations in other proteins that influence *Notch* activity, such as *sel-10* homologs.

Materials and Methods

General methods and strains

General methods are described by Brenner (1974). The wild-type parent for all strains was *C. elegans* var. Bristol strain N2. Mapping experiments utilized the Bristol/Bergerac congenic strain

GS352, in which the region between *rol-4* and *par-1* of Bristol was replaced with the corresponding region from the Bergerac strain BO (Tuck and Greenwald, 1996). Strains were grown at 20° unless otherwise noted. Mutations used are described in Hodgkin (1997); additional references for critical alleles are also given. Note that all genetic constructions containing *sel-10* used a *sel-10* chromosome that does not have the linked modifier mutation *arX* (Sundaram and Greenwald, 1993).

LGIII: *dpy-17(e164)*, *unc-36(e251)* and *unc-32(e189)* ; *lin-12(ar170)* (Hubbard et al., 1986; E.J.A.H., unpublished observations); *lin-12(n379)* (Greenwald et al., 1983).

LGIV: *dpy-20(e1282)* .

LGV: *nDf42* (M. Hengartner and H.R. Horvitz, personal communication), *lon-3(e2175)*, *rol-4(sc8)*, *sel-10(ar41)* (Sundaram and Greenwald, 1993), *him-5(e1490)*, *unc-76(e911)*.

Mapping of the sel-10 *locus*

sel-10 had been genetically mapped between *lin-25* and *unc-76* V (Sundaram and Greenwald, 1993), and approximately 0.2MU to the left of *him-5* (data not shown). We mapped *sel-10* between *arP3* and *TCPAR1* by identifying Rol Him non-Unc recombinants from heterozygotes of the genotype *rol-4 BO unc-76/lon-3 sel-10 him-5* constructed using the strain GS352. Fifty independent recombinants were analyzed by Southern blot hybridization for the presence of *arP3* and *TCPAR1* (see Tuck and Greenwald, 1996), and each recombinant strain was tested for the presence of *sel-10(ar41)* by crossing into *lin-12(n379)* and scoring for the Muv phenotype. Mapping data can be found in ACeDB (Edgley et al. 1997).

sel-10 *cloning by anti-suppression assay*

sel-10(ar41) partially suppresses the 2AC defect caused by *lin-12(ar170)*: at 25°, ~80% of *lin-12(ar170)* animals have 2AC while ~25% of *lin-12(ar170); sel-10(ar41)* animals have 2AC. We used reversal of suppression as the basis of assessing *sel-10(+)* activity of microinjected DNAs. Transgenic lines were generated by microinjecting the germ lines of *lin-12(ar170); dpy-*

20(e1282);sel-10(ar41) him-5(e1490) hermaphrodites with cosmid or plasmid DNA (Mello et al., 1991) at a concentration of 5μg/ml, along with the *dpy-20(+)* transformation marker DNA at 10μg/ml (plasmid pMH86; Han and Sternberg, 1991) and carrier Bluescript DNA (Stratagene) at 90μg/ml. Synchronous populations were obtained by allowing groups of transgenic hermaphrodites to lay eggs at 20° for 1-2 hours and tranferring the eggs to 25°. The non-Dpy L3 hermaphrodites were then scored for the number of anchor cells. The injected tester DNA was considered to contain *sel-10(+)* sequences if >50% of the non-Dpy animals had 2AC. Typically, 60-80% 2AC was achieved in these "rescued" lines. Some arrays scored as having *sel-10(+)* activity were subjected to a second test, the ability to reverse the Muv phenotype of *lin-12(n379);sel-10 (ar41)*. Initial rescue was obtained with a pool of seven overlapping cosmids from the region (each at 5μg/ml), then with the single cosmid C07E11, and then with plasmids derived from C07E11, as shown in Figure 1.

Plasmids containing sel-10 *genomic sequences*

pJH151 was constructed by digesting cosmid C07E11 with *Bam*HI and ligating the 15kb fragment to Bluescript KS+ (Stratagene). pJH166 was constructed by ligating an 8kb *Pst*I-*Sal*I fragment from pJH151 into Bluescript KS+. The *Pst*I site was from the vector, while the *Sal*I site is from the genomic sequences. The ~9kb *Sal*I fragment was removed from pJH151 to form pJH165, and pJH167 was made by ligation of the internal *Hin*dIII fragment of pJH151 into Bluescript. To construct pJH169, pJH166 was cut with *Pme*I and a linker containing an *Nhe*I site with a stop codon in all frames (NEB #1060) was inserted, creating a stop codon after amino acid 172 in the SEL-10 sequence.

sel-10 *overexpression*

arEx93 was generated by microinjecting *dpy-20* hermaphrodites with pJH166 [*sel-10(+)*] at a concentration of 100μg/ml and pMH86 [*dpy-20(+)*] at 10μg/ml. Strains carrying this array segregate sterile animals as well as fertile animals; the basis for the sterility has not been

established. Many of the fertile animals display a leaky Egl phenotype similar to that observed in certain *lin-12* hypomorphic mutants. Qualitatively similar results were observed with other lines at this concentration and with lines established using pJH166 at 50μg/ml (data not shown).

The control array *arEx149* was established by microinjecting *unc-32; dpy-20* hermaphrodites with pMH86 at 10μg/ml, and Bluescript DNA at 90μg/ml into *unc-32; dpy-20* animals.

Molecular analysis of sel-10

Standard methods were used for the manipulation of recombinant DNA (Sambrook et al., 1989). *sel-10(+)* cDNAs were obtained by screening approximately 100,000 pfu from a phage library kindly provided by R. Barstead (Barstead and Waterston, 1989). Ten positive plaques were purified by two subsequent rounds of screening using a radiolabelled fragment from pJH166 (~8 kb *Bam*HI/*Sal*I fragment) as a probe. cDNA 1A, the longest cDNA obtained, was sequenced in its entirety on one strand and compared with genomic sequence from the genome project using GENEFINDER (see Waterston et al., 1997). The sequence of the cDNA 1A differed from the GENEFINDER prediction in the location of the junction between the second and third exons and in the predicted 3' end. Four of the cDNAs were polyadenylated at their 3' ends (one 294, one 581 and the other two 601 bases after the predicted stop codon). Of these, only the last two were in the context of a conserved polyadenylation signal. The 5'-most cDNA end was located in codon 1 (cDNA 8 begins at G of the first ATG), but a PCR product was amplified from DNA prepared from the same cDNA library (Barstead and Waterston, 1989; C. Dong, personal communication) contained the SL1 spliced leader at the predicted sequence 4 bases 5' of the first ATG. The 22 base SL1 sequence and a primer straddling the 5th and 6th exons were used for the 5' end amplification.

Sequence analysis

Standard techniques were used to obtain sequence of the 1A cDNA (Sambrook et al., 1989). The lesions associated with the *sel-10(ar41)* and *sel-10(ar28)* mutations were found by direct sequencing of two PCR products from single-stranded templates (Allard et al., 1991; Kaltenboeck et al., 1992), using internal primers to cover the entire region. One small segment was subcloned and sequenced (from two independent reactions each), as the sequence from this region was not easily generated using the direct method. Sequence comparisons and alignments were obtained using Blast (Altschul et al., 1990) through the NCBI web site and GCG (version 8, Devereux et al., 1984) programs.

Plasmids for cell culture experiments

Plasmids used in the transient transfection experiments were constructed in pLNCX (Miller et al., 1989) or pQNCX (Qingyou Yan and J.K., unpublished observations), vectors that drive gene expression under the control of a CMV promoter.

pQNClacZ contains the bacterial *lacZ* gene.

pQNClin-12(intra)HA encodes a protein with a methionine-containing hemagglutinin epitope (Wilson et al., 1984) fused in frame N-terminal to LIN-12(intra) at amino acid 939.

pLNCint-3 contains cDNA corresponding to the *Notch4* region expressed in the *int3* insertion, beginning at amino acid 1411. The Notch4(int3) protein includes the entire intracellular domain of Notch4 and additional sequences (see Uyttendaele et al., 1996).

pQNCsel-10myc (pJH186) encodes a protein with six myc epitope tags (Roth et al., 1991) fused in frame to cDNA 1A at amino acid 13 of SEL-10.

pQNCsel-10HA (pJH184) encodes a protein with a methionine-containing hemagglutinin epitope from pACT2 (Durfee et al., 1993) fused in frame (along with a short stretch of polylinker) to cDNA 1A at amino acid 13.

Transfection , Immunoprecipitations, and Western Blot Analysis

293T (Bosc23) cells (Pear et al., 1993) were maintained in DMEM with 10% fetal bovine serum (FBS). A confluent plate of cells was split 1:3 the day prior to transfection. For one 60mm plate of cells, 4μg of each plasmid DNA was transfected using the calcium phosphate precipitation method. The total amount of DNA was kept constant by supplementation with vector DNA or lacZ-containing plasmids.

Two days after transfection, cells were harvested and lysed in TENT buffer (50mM Tris·Cl (pH8.0), 2mM EDTA, 150mM NaCl, 1% Triton-X 100) containing protease inhibitors (2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml pepstatin, 0.5mM PMSF). Lysates were clarified by centrifugation at 10,000g for 10 minutes and protein content was determined using the BioRad Protein determination kit and samples were normalized for protein content. Extracts were precleared with sepharose CL-4B beads, incubated with antibodies (3μl of anti-Notch4 antiserum, 50μl of 12CA5 anti-HA supernatant, or 200 μl of 9E10 anti-myc supernatant) for six hours at 4°C, then incubated with 40μl of 50% slurry of protein A-sepharose for 1 hour at 4°C. The protein A-sepharose beads were washed with TENT buffer three times by vortexing for 10 minutes, beads were boiled in 30μl 1X protein loading buffer, and then electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The blot was blocked overnight at 4°C with TBST (10mM Tris, pH 8.0, 150mM NaCl, 0.2% Tween 20) containing 1% bovine serum albumin (TBST-BSA). The blot was then incubated with 1° antibody diluted (1:2,000 anti-Notch4; 1:50 for 12CA5; 1:10 for 9E10) in TBST-BSA for 1 hour, washed three times for 5 minutes each with TBST, and incubated with 2° antibody in TBST-BSA for 1 hour. After three washes, the signal was visualized by chemiluminescence (Amersham, ECL).

The anti-Notch4 antiserum (G.W. and J.K., unpublished observations) is directed against the C-terminal region of Notch4 (residues 1788-1964) (Uyttendaele et al., 1996). 12CA5 anti-HA antibody was obtained from Berkeley Antibody Co., Richmond, CA. 9E10 anti-myc antibody was prepared from culture supernatants of the 9E10 hybridoma (Evan et al., 1985).

Acknowledgements

We are grateful to Hendrik Uyttendaele for Notch4(int3) constructs, Qingyou Yan for expression vectors and Gary Struhl for plasmids and epitope tags. We also thank Denise Brousseau and Richard Ruiz for technical assistance; Naiche Adler, Conrad Leung, Ilya Temkin and Jin Yu for help with genetics; and the Caenorhabditis Genetics Center and Michael Koelle for providing strains. We are indebted to Edward Kipreos, Mark Goebl and Ray Deshaies for sharing information and ideas prior to publication, Simon Tuck and Tim Schedl for helpful advice, and Barth Grant, Diane Levitan, Alicia Melendez, Chenhui Wen, Stevan Hubbard and Gary Struhl for valuable discussions and comments on the manuscript. This work was supported by the Damon Runyon-Walter Winchell Cancer Research Fund (E.J.A.H.), NIH grant GM37602 (I.G.) and the US Army Medical Research and Material Command (USAMRMC) under grant DAMD17-94-J-4410 (J.K.). E.J.A.H. is a Postdoctoral Associate and I.G. is an Associate Investigator of the Howard Hughes Medical Institute.

References

Altschul, S.F., W. Gish, W. Miller, E.W. Meyers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Aroian, R.V., M. Koga, J.E. Mendel, Y. Ohshima, and P.W. Sternberg. 1990. The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**: 693-9.

Artavanis-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. Notch signaling. *Science* **268**: 225-235.

Austin, C.P., D.E. Feldman, J.A. Ida, and C.L. Cepko. 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of *Notch*. *Development* **121**: 3637-3650.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl, J.W. Harper, and S.J. Elledge. 1996. *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**: 263-274.

Barstead, R.J., and R.H. Waterston. 1989. The basal component of the nematode dense-body is vinculin. *J. Biol. Chem.* **264**: 10177-10185.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71-94.

Cagan, R.L., and D.F. Ready. 1989. Notch is required for successive cell decisions in the developing Drosophila retina. *Genes Dev.* **3**: 1099-1112.

Chitnis, A., D. Henrique, J. Lewis, D. Ish-Horowicz, and C. Kintner. 1995. Primary neurogenesis in *Xenopus* embryos regulated by a homolog of the *Drosophila* neurogenic gene *Delta*. *Nature* **375**: 761-766.

Christensen, S., V. Kodoyianni, M. Bosenberg, L. Friedman, and J. Kimble. 1996. *lag-1*, a gene required for *lin-12* and *glp-1* signaling in *C. elegans*, is homologous to human CBF1 and *Drosophila* Su(H). *Development* **122**: 1373-1383.

Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell* **79**: 13-21.

Ciechanover, A., and A.L. Schwartz. 1994. The ubiquitin-mediated proteolytic pathway: mechanisms of recognition of the proteolytic substrate and involvement in the degradation of native cellular proteins. *FASEB J.* **8**: 182-191.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**: 387-395.

DeVore, D.L., H.R. Horvitz, and M.J. Stern. 1995. An FGF receptor signaling pathway is required for the normal cell migrations of the sex myoblasts in *C. elegans* hermaphrodites. *Cell* **83**: 611-620.

Drexler, H.G., R.A.F. MacLeod, A. Borkhardt, and J.W.G. Janssen. 1995. Recurrent chromosomal translocations and fusion genes in leukemia-lymphoma cell lines. *Leukemia* **9**: 480-500.

Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, A.E. Kilburn, W.-H. Lee, and S.J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7**: 1-12.

Ellisen, L.W., J. Bird, D.C. West, A.L. Soreng, T.C. Reynolds, S.D. Smith, and J. Sklar. 1991. *TAN-1*, the human homolog of the Drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**: 649-661.

Evan, G.I., Lewis, G.K., Ramsay, G. and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**: 3610-3616.

Fields, S., and O.-k. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature* **340**: 245-246.

Gallahan, D., and R. Callahan. 1987. Mammary tumorigenesis in feral mice: identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J. Virol.* **61**: 66-74.

Gaudet, R., A. Bohm, and P.B. Sigler. 1996. Crystal structure at 2.4 A resolution of the complex of transducin βγ and its regulator, phosducin. *Cell* **87**: 577-568.

Grant, B., and I. Greenwald. 1997. Structure, function and expression of SEL-1, a negative regulator of LIN-12 and GLP-1 in *C. elegans*. *Development* **124**: 637-644.

Greenwald, I. 1994. Structure/function studies of lin-12/Notch proteins. *Curr. Opin. Gen. Dev.* **4**: 556-562.

Greenwald, I., and G. Seydoux. 1990. Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. *Nature* **346**: 197-9.

Greenwald, I.S., P.W. Sternberg, and H.R. Horvitz. 1983. The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. *Cell* **34**: 435-444.

Han, M., and P.W. Sternberg. 1991. Analysis of dominant-negative mutations of the Caenorhabditis elegans let-60 ras gene. *Genes & Dev.* **5**: 2188-98.

Heitzler, P., and P. Simpson. 1991. The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**: 1083-1092.

Kimble, J. 1981. Alteration in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol.* **87**: 286-300.

Kimble, J., and D. Hirsh. 1979. The post-embryonic cell lineages of the hermaphrodites and male gonads in *Caenorhabditis elegans*. *Dev. Biol.* **87**: 396-417.

King, R.W., R.J. Deshaies, J.-M. Peteres, and M.W. Kirschner. 1996. How proteolysis drives the cell cycle. *Science* **274**: 1652-1658.

Kipreos, E.T., L.E. Lander, J.P. Wing, W.W. He, and E.M. Hedgecock. 1996. *cul-1* is required for cell cycle exit in *C. elegans* and identifies a novel gene family. *Cell* **85**: 829-839.

Krause, M., and D. Hirsh. 1987. A *trans*-spliced leader sequence on actin mRNA in *C. elegans*. *Cell* **49**: 753-761.

Kumar, A., and J.V. Paietta. 1995. The sulfur controller-2 negative regulatory gene of Neurospora crassa encodes a protein with beta-transducin repeats. *Proc. Natl. Acad. Sci. (USA)* **92**: 3343-3347.

Lambright, D.G., J. Sondek, A. Bohm, N.P. Skiba, H.E. Hamm, and P.B. Sigler. 1996. The 2.0 A crystal structure of a heterotrimeric G protein. *Nature* **379**: 311-319.

Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M.W. Young. 1993. Antineurogenic phenotypes induced by truncated *Notch* proteins indicate a role in signal transduction and may point to a novel function for *Notch* in nuclei. *Genes Dev.* **7**: 1949-1965.

Mango, S.E., E.M. Maine, and J. Kimble. 1991. Carboxy-terminal truncation activates glp-1 protein to specify vulval fates in Caenorhabditis elegans. *Nature* **352**: 811-815.

Mathias, N., S.L. Johnson, M. Winey, A.E.M. Adams, L. Goetsch, J.R. Pringle, B. Byers, and M.G. Goebl. 1996. Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S phase transition and identifies a conserved family of proteins. *Mol. Cell Biol.* **16**: 6634-6643.

Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**: 3959-3970.

Natorff, R., M. Balinska, and A. Paszewski. 1993. At least four regulatory genes control sulphur metabolite repression in Aspergillus nidulans. *Mol. Gen. Genet.* **238**: 185-192.

Neer, E.J., C.J. Schmidt, R. Nambudripad, and T.F. Smith. 1994. The ancient regulatory-protein family of WD-repeat proteins. *Nature* **371**: 297-300.

Nellen, D., R. Burke, G. Struhl, and K. Basler. 1996. Direct and long range action of a DPP morphogen gradient. *Cell* **85**: 357-368.

Pear, W.S., G.P. Nolan, and D. Baltimore. 1993. Production of high-titre helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. (USA)* **90**: 8392-8396.

Robbins, J., B.J. Blondel, D. Gallahan, and R. Callahan. 1992. Mouse mammary tumor gene *int-3*: a member of the *Notch* gene family transforms mammary epithelial cells. *J. Virol.* **66**: 2594-2599.

Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**: 364-368.

Roth, M.B., Zahler, A., and J.H. Stolk. 1991. A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. *J. Cell Biol.* **115:** 587-596.

Seydoux, G., and I. Greenwald. 1989. Cell autonomy of *lin-12* function in a cell fate decision in *C. elegans*. *Cell* **57**: 1237-45.

Sondek, J., A. Bohm, D.G. Lambright, H.E. Hamm, and P.B. Sigler. 1996. Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 A resolution. *Nature* **379**: 369-374.

Struhl, G., K. Fitzgerald, and I. Greenwald. 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**: 331-45.

Sundaram, M., and I. Greenwald. 1993. Suppressors of a *lin-12* hypomorph define genes that interact with both *lin-12* and *glp-1* in *Caenorhabditis elegans*. *Genetics* **135**: 765-783.

Thomas, D., L. Kuras, R. Barbey, H. Cherest, P.L. Blaiseau, and Y. Surdin-Kerjan. 1995. Met30p, a yeast transcriptional inhibitor that responds to S-adenosylmethionine, is an essential protein with WD40 repeats. *Mol. Cell. Biol.* **15**: 6526-6534.

Tuck, S., and I. Greenwald. 1995. *lin-25*, a gene required for vulval induction in *Caenorhabditis elegans*. *Genes Dev.* **9**: 341-357.

Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. 1996. *Notch4/int-3*, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene. *Development* **122**: 2251-2259.

Washburn, T., E. Schweighoffer, T. Gridley, D. Chang, B.J. Fowlkes, D. Cado, and E. Robey. 1997. *Notch* activity influences the ab versus gd T cell lineage. *Cell* **88**: 833-843.

Wilkinson, H.A., K. Fitzgerald, and I. Greenwald. 1994. Reciprocal changes in expression of the receptor *lin-12* and its ligand *lag-2* prior to commitment in a *C. elegans* cell fate decision. *Cell* **79**: 1187-1198.

Wilson, I.A., Niman, H.L, Houghten, R.A., Cherenson, A.R., Connolly, M.L. and R.A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell* **37**: 767-778.

Figure legends

Figure 1. Molecular cloning of *sel-10*. See Materials and Methods for details of rescue assays, plasmid constructions, and molecular analysis. Genetic markers used to map *sel-10* are italicized, and two cosmids that contain *sel-10* sequences are shown in the box. The cosmid C07E11 and derivatives were tested for rescue. pJH169 is identical to pJH166 except that it contains a stop codon (indicated by an asterisk) in the predicted coding sequence after codon 172. Restriction sites are B, *Bam*HI; H, *Hin*dIII; S, *Sal*I.

Figure 2. cDNA sequence and predicted protein product of *sel-10*. Splice junctions are indicated by arrows below the DNA sequence. The first arrow indicates the SL1 splice junction. The F-Box (Kumar and Paietta, 1995, Bai et al., 1996) and the WD40 repeats are overlined and labelled in the Figure. The lesions in *sel-10(ar41)* and *sel-10(ar28)* are indicated with reverse contrast letters in the nucleotide sequence and a bold asterisk above the amino acid; both are G to A transitions resulting in W to stop codon changes in the amino acid sequence at residues 323 and 511, respectively. The cDNA termination codon is marked with an asterisk. A sequence conforming to the consensus polyadenylation signal sequence is underlined, and sites of polyA attachment are marked in bold. Two independent cDNAs contained polyA fourteen nucleotides downstream of this signal; two alternative sites of attachment were also observed. Sequence information has been submitted to Genbank (accession number AF020788).

Figure 3: SEL-10 is a member of the CDC4 family of F-box/WD40-repeat proteins. The CDC4 sequence (accession number X05625) is from Genbank. There are other potential CDC4 family members in the database; limited sequence data for a potential SEL-10 ortholog is given in part D. For further discussion of the CDC4 family, see Kumar and Paietta (1995) and Bai et al. (1996). Reverse contrast letters indicate amino acid identity.

A) Schematic depiction of SEL-10 and CDC4, drawn to scale. The percentage of identical amino acids in each region is indicated. Members of the CDC4 family all have this general

organization, with some variability in the length and sequence of their amino and carboxy termini.

B) Alignment of SEL-10 and CDC4 F-Boxes.

C) Alignment of WD40 repeats from SEL-10 and CDC4.

D) Alignment of SEL-10 and a potential human ortholog. Partial sequence of a human cDNA encoding a sequence highly similar to the C terminus of SEL-10 was obtained from Genbank (accession number H22962) and was extended into the WD40 repeats by direct sequencing of clone ym50h08.s1 (G.W. and J.K., unpublished observations). The predicted amino acid sequence encoded by the available human cDNA sequence is shown. The database also contains partial sequence information for a rat cDNA (Genbank accession number H34371) that is predicted to encode a peptide that is 100% identical to the last 35 amino acids of the available human sequence (data not shown).

Figure 4: Coimmunoprecipitation of *C. elegans* SEL-10 with either *C. elegans* LIN-12(intra) or murine Notch4(int3) from transfected 293T cells.

(A) Samples were immunoprecipitated with anti-HA antibody and the Western blot was probed with anti-myc to visualize SEL-10myc (top panel) or anti-HA to visualize LIN-12(intra)HA (bottom panel). Arrow indicates the expected mobility of SEL-10myc.

(B) Samples were immunoprecipitated with anti-myc antibody and the Western blot was probed with anti-HA (top panel) or anti-myc (bottom panel). Arrow indicates the expected mobility of LIN-12(intra)HA.

(C) Samples were immunoprecipitated with anti-Notch4 antibody and the Western blot was probed with anti-HA to visualize SEL-10HA (top panel) or anti-Notch4 to visualize Notch4(int3) (bottom panel). Arrow indicates the expected mobility of SEL-10HA.

(D) Samples were immunoprecipitated with anti-HA antibody and the Western blot was probed with anti-Notch4 (top panel) or anti-HA (bottom panel). Arrow indicates the expected mobility of Notch4(int3).

For details see Materials and Methods. Lane 1, pQNClacZ. Lane 2, pQNClin-12(intra)HA + pQNClacZ. Lane 3, pQNClacZ + pQNCsel-10myc. Lane 4, pQNClin-12(intra)HA + pQNCsel-10myc. Lane 5, mock transfected cells. Lane 6, pLNCint3 + pQNCX. Lane 7, pLNCX + pQNCsel-10HA. Lane 8, pLNCint3 + pQNCsel-10HA.

Table 1. *sel-10* gene dosage analysis.

Relevant genotype	%Egl (n)	%Muv (n)	%Ste/Let (n)
20°			
lin-12(d)/+[a]	6 (93)	0 (93)	0 (93)
lin-12(d)/+;sel-10[b]	91 (54)	0 (54)	0 (54)
lin-12(d)/+;sel-10/Df[c]	92 (39)	15 (39)	0 (39)
15°			
lin-12(d); +[d]	86 (60)	0 (60)	0 (60)
lin-12(d);sel-10/+[e]	98 (62)	0 (62)	0 (62)
lin-12(d);+/Df[f]	89 (57)[i]	62 (74)	10 (63)
lin-12(d);sel-10[g]	100 (70)[i]	78 (197)	55 (126)
lin-12(d);sel-10/Df[h]	–	85 (34)	100 (34)[j]

Complete genotypes are:

[a]*lin-12(n379)/unc-36(e251); lon-3(e2175)/him-5(e1490)*

[b]*lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)*

[c]*lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42*

[d]*lin-12(n379); lon-3(e2175)/him-5(e1490)*

[e]*lin-12(n379); lon-3(e2175) sel-10(ar41)/him-5(e1490)*

[f]*lin-12(n379); lon-3(e2175) /nDf42*

[g]*lin-12(n379); lon-3(e2175) sel-10(ar41)*

[h]*lin-12(n379); lon-3(e2175) sel-10(ar41)/nDf42*

Complete broods were scored by picking individual L4 animals and inspecting the plates at 24 and 48 hours for the absence of eggs on the plate (Egl) and for the presence of three or more pseudovulvae along the ventral hypodermis (Muv). Plates

were then inspected after an additional three days for the presence of live progeny ("Ste/Let" refers to absence of live progeny and was, in this case, a combination of sterility (Ste) and embryonic lethality(Let)). In some cases, broods were scored in batch for the Muv phenotype.

[i]Percent of fertile animals displaying the Egl defect.

[j]Inferred genotype: Complete broods from *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42)* were scored. The percentage of sterile non-Unc, non-Lon progeny (34/97 = 35%) is approximately equal to that expected for *lin-12(n379);lon-3(e2175) sel-10(ar41)/nDf42* genotypic class. Of the remaining 63 animals, 61/63 were unambiguously scored as heterozygotes in the next generation while the remaining 2/63 did not have a sufficient number of progeny to score unambiguously.

Table 2. Increased dosage of *sel-10* reduces *lin-12* activity.

A. Suppression of phenotypes associated with increased *lin-12* activity

Relevant genotype	%0AC (n)
lin-12(d); dpy-20;Ex[sel-10(+) dpy-20(+)]	51 (47)
lin-12(d); dpy-20;Ex[dpy-20(+)]	95 (44)

B.Enhancement of phenotypes associated with reduced *lin-12* activity

Relevant genotype	%2AC (n)
lin-12(h); dpy-20;Ex[sel-10(+) dpy-20(+)]	97 (34)
lin-12(h); dpy-20;Ex[dpy-20(+)]	30 (40)
lin-12(+); dpy-20(e1282);Ex[sel-10(+) dpy-20(+)]	0 (87)

Complete genotypes:*lin-12(d)=lin-12(n379), lin-12(h) = lin-12(ar170), dpy-20(h) = dpy-20(e1282), Ex[sel-10(+) dpy-20(+)]=arEx93, Ex[dpy-20(+)]=arEx149.* (See Materials and Methods for details of strain constructions). Non-Dpy animals segregating from the strains were scored in the L3 stage for the number of anchor cells.

Table 3. Cell autonomy of *sel-10* function.

A.Enhancement of *lin-12(intra)*

Relevant genotype	%Egl (n)	%Mig (n)
sel-10(+);arEx[lin-12(intra)]	16 (88)[a]	10 (57)
sel-10(ar41);arEx[lin-12(intra)]	46 (136)[a]	59 (90)

B.Cell Ablation

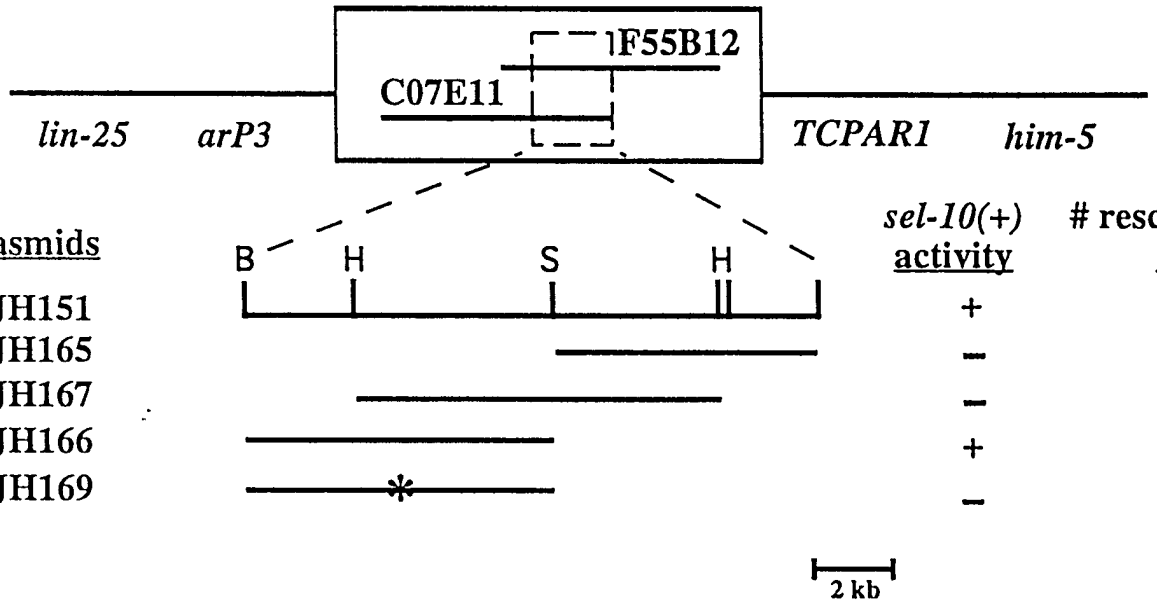
	%0AC (n)	
Relevant genotype	unoperated	operated
lin-12(n379)/+ ; sel-10(+)	10 (57)	9 (11)
lin-12(n379)/+; sel-10(ar41)	97 (71)	83 (12)

A. All strains also contained *him-5(e1490). arEx[lin-12(intra)]* = *arEx152* (Fitzgerald, personal communication) is an extrachromosomal array formed by microinjection (Fire, 1986; Mello et al., 1991) of pRF4 (plasmid containing *rol-6(su1006)* sequence that confers a Rol phenotype onto worms carrying the array) at 100µg/ml and pLC8 (Struhl et al., 1993).

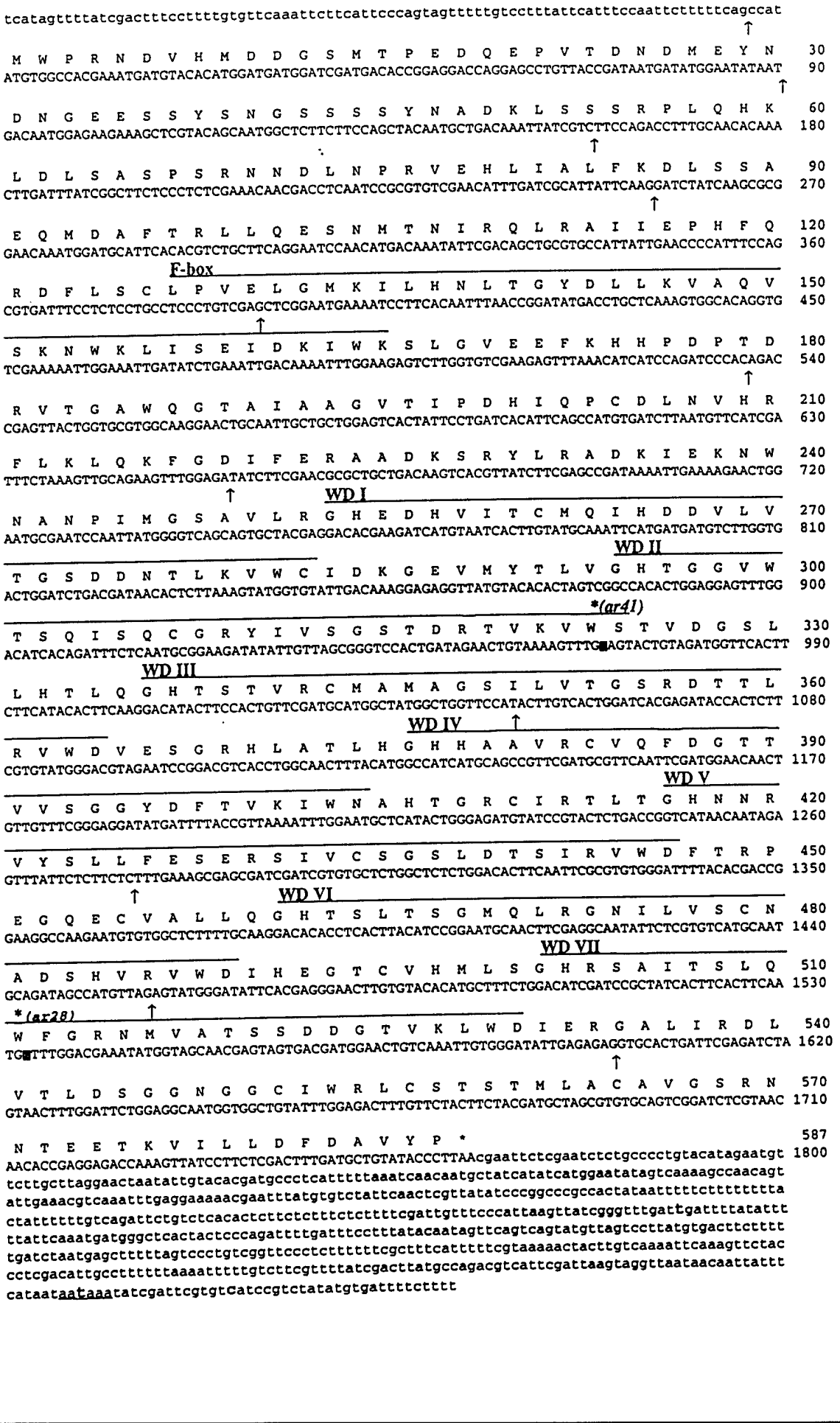
[a] We infer that these Egl hermaphrodites lacked an AC because we scored additional hermaphrodites of relevant genotype *sel-10; arEx[lin-12(intra)]* in the L3 stage for the presence or absence of an AC and as adults for their egg-laying ability, and found that nine hermaphrodites that clearly had a single AC were non-Egl, while nine hermaphrodites that clearly lacked an AC were Egl.

B.Complete genotype: *dpy-17(e164) lin-12(n379)/unc-32(e189); lon-3(e2175) sel-10(+ or ar41)*

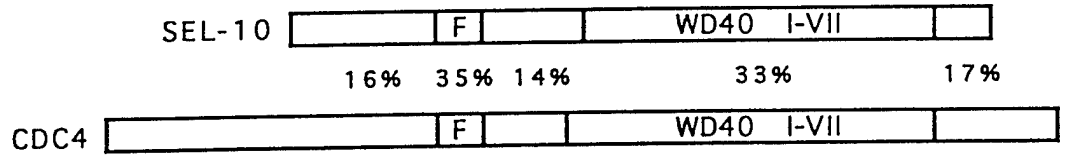
"Operated" refers to worms in which Z4 was laser ablated in the early L1 stage (when the gonad primordium consisted of four cells, Z1-Z4). Worms were then scored in the L3 stage for the presence or absence of an AC.



Plasmids	*sel-10*(+) activity	# rescued lines/ total
pJH151	+	2/2
pJH165	–	0/5
pJH167	–	0/3
pJH166	+	3/3
pJH169	–	0/2


F-box
WD I
WD II
*(ar41)
WD III
WD IV
WD V
WD VI
WD VII
*(ar28)

A. Schematic representation of SEL-10 and CDC4



B. Alignment of F-Box

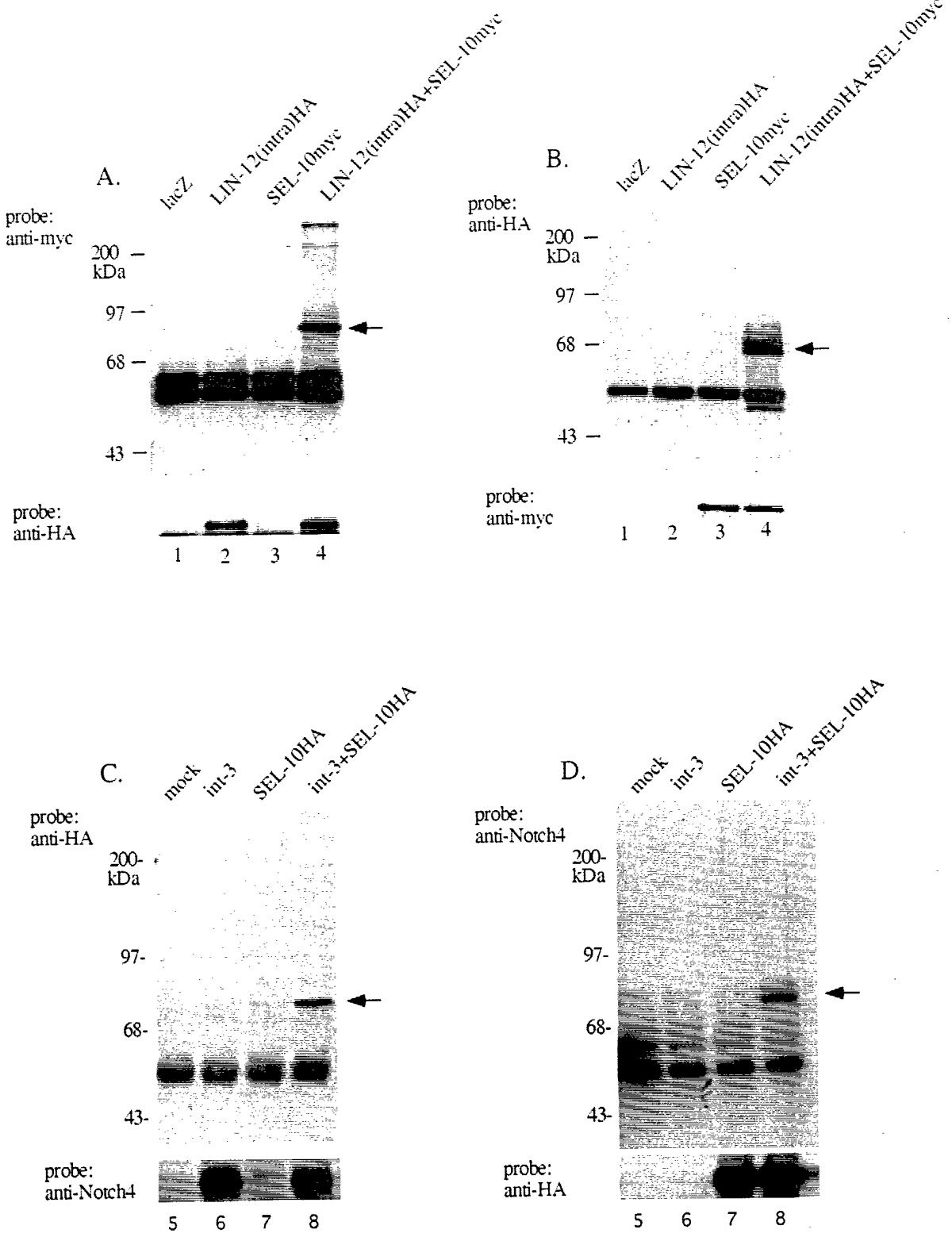
```
SEL-10   127   LPVELGMKILHNLTGYDLLKVAQVSKNW.KLISEIDKIWKSLG
CDC4     278   LPFEISLKIFNYLQFEDIINSLGVSQNWNKIIRKSTSLWKKLL
```

C. Alignment of WD40 repeats

```
I     SEL-10   253   GHEDHVITCMQIHDDVLVTGSDDNTLKVWC
      CDC4     419   GHMTSVITCLQFEDNYVITGADDKMIRVYD

II    SEL-10   294   GHTGGVWTSQISQCGRYIVSGSTDRTVKVWS
      CDC4     460   GHDGGVWALKYAHGG.ILVSGSTDRTVRVWD

III   SEL-10   336   GHTSTVRCMAMAG....SILVTGSRDTTLRVWD
      CDC4     502   GHNSTVRCLDIVEYKNIKYIVTSGRDNTLHVWK

IV    SEL-10   376   GHHAAVRCVQFDGTTVVSGGYDFTVKIWN
      CDC4     569   GHMASVRTVSGHGNIVVSGSYDNTLIVWD

V     SEL-10   416   GHNNRVYSLLFESERSIVCSGSLDTSIRVWD
      CDC4     609   GHTDRIYSTIYDHERKRCISASMDTTIRIWD

VI    SEL-10   461   GHTSLTSGMQLRGNILVSCNADSHVRVWD
      CDC4     671   GHTALVGLLRLSDKFLVSAAADGSIRGWD

VII   SEL-10   501   GHR..SAITSLQWFGRNMVATSSDDGTVKLWD
      CDC4     710   HHTNLSAITT.FYVSDNILVSGSENQFN.IYN
```

D. Alignment of partial sequence of potential human SEL-10 ortholog

```
                    WD VI                                WD VII
Ce SEL-10 480   NADSHVRVWDIHEGTCVHML...SGHRSAITSLQWFGRNMVATSSDDGTV
Hs SEL-10       NADSTVKIWDIKTGQCLQTLQVPNKHQSAVTCLQ.FNKNFVITSSDDGTV

                ____
Ce SEL-10 527   KLWDIERGALIRDLVTLDSGGNGGCIWRLCSTSTMLACAVGSRNNTEETK
Hs SEL-10       KLWDLKTGEFIRNLVTLESGGSGGVVWRIRASNTKLVCAVGSRNGTEETK

Ce SEL-10 577   VILLDFD
Hs SEL-10       LLVLDFD
```


A.
lacZ
LIN-12(intra)HA
SEL-10myc
LIN-12(intra)HA+SEL-10myc
probe:
anti-myc
200 –
kDa
97 –
68 –
43 –
probe:
anti-HA
1 2 3 4
B.
lacZ
LIN-12(intra)HA
SEL-10myc
LIN-12(intra)HA+SEL-10myc
probe:
anti-HA
200 –
kDa
97 –
68 –
43 –
probe:
anti-myc
1 2 3 4
C.
mock
int-3
SEL-10HA
int-3+SEL-10HA
probe:
anti-HA
200-
kDa
97-
68-
43-
probe:
anti-Notch4
5 6 7 8
D.
mock
int-3
SEL-10HA
int-3+SEL-10HA
probe:
anti-Notch4
200-
kDa
97-
68-
43-
probe:
anti-HA
5 6 7 8